THE STRUCTURAL PROTEINS OF INFLUENZA VIRUS

by

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Addendum

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STATEMENT.

The work presented in this addendum is based on my own ideas and was performed entirely by myself with no supervision at the Commonwealth Serum Laboratories, Parkville, Victoria, except as indicated in the Acknowledgements.

I.P. Griffith.

Ian Peter Griffith.

ACKNOWLEDGEMENTS.

I am greatly indebted to the Commonwealth Serum Laboratories for generous financial support and for allowing me the use of their facilities to carry out the work presented in this addendum; in particular, I should like to thank Dr. Frank Warburton for encouraging this work.

I am also grateful to Professors D.O. White and S.J. Leach, and Drs. Elizabeth Haslam and I.M. Cheyne for helpful discussions; to Messrs. N.G. Wrigley, M. Taylor and R. Hamilton for the electron micrographs; to Mr. J. Ride for the computer analysis; to Dr. B. Milligan for the gifts of dyes; and to Drs. D.C. Shaw and W.H. Sawyer for gifts of proteins.

PREFACE.

This addendum is presented in the form of papers prepared for publication; hence, the figure numbering for each paper applies to that paper only. Additional references have been listed at the end of the addendum. The abbreviations used were generally those recommended by the Biochemical Journal (Biochem. J. (1967) 102, 1) or have been listed previously (previous submission, p.ix).

TABLE OF CONTENTS.

		Page
STATEMENT		i
ACKNOWLEDGEMENTS		ii
PREFACE		iii
TABLE OF CONTENTS		iv
GENERAL INTRODUCTION TO THE ADDENDUM		1
PAPER 1.	The fine structure of influenza virus.	10
PAPER 2.	Immediate visualisation of protein in dodecyl sulphate-polyacrylamide gels by prestaining with a Remazol dye.	34
PAPER 3.	The preparation of highly infectious influenza virus.	47
PAPER 4.	The effect of cystine cross-links on the mobility of proteins in dodecyl sulphate-polyacrylamide gels.	61
PAPER 5.	The polypeptides of the influenza virion.	81
PAPER 6.	A simple procedure for the recovery of protein from preparative gel slabs.	99
GENERAL DISCUSSION OF RESULTS.		101
REFERENCES		110

INTRODUCTION

Earlier work (previous submission) described attempts to prepare a specific antiserum to 'the' "soluble antigen" (chapter 1) and to purify 'it' from infected tissue sources (chapter 2). Chapter 3 was concerned with purification of virus, chapter 4 described attempts to disrupt the virion, and to separate and identify the split products. Chapter 5 was concerned with analysis of some isolated protein fractions.

Conclusions from the earlier work were based on a number of assumptions; among these were:

1) that only a single internal antigen was present in the virion, the RNP, and that specific antiserum could be prepared against this by injecting crude concentrates of infected tissues into rabbits (Chapters 1 and 2); also that the polypeptide R was derived from the RNP (Chapters 4 and 5).

2) that erythrocyte adsorption-elution was the best method of initial concentration and purification of the BEL strain (Chapter 3).

3) that the pure virus was completely separated into its constituent polypeptides by oxidation with performic acid followed by polyacrylamide gel electrophoresis in the presence of 8 M urea and SDS.

4) that the 8% polyacrylamide gel electrophoresis technique, both analytical and preparative, satisfactorily separated the viral polypeptides into three major species.

This addendum is presented as six papers (the last of which is only summarised), all of which are in preparation for publication.

One only (paper 1) is in conjunction with another worker (N.G. Wrigley). The work reported is largely new work, undertaken for a variety of reasons.

Paper 1 is concerned with the fine structure of the virion and its structural proteins as revealed by the electron microscope. This paper embodies work reported earlier (Chapter 4) and is expanded to include new observations. The two most pertinent to the previous work are the observation of an additional internal component in the virion, and that the head of the neuraminidase may have a molecular weight of the order of 64,000 consisting of 4 subunits of molecular weight 16,000.

Paper 2 describes a new technique for staining proteins with reactive dyes of the Remazol type prior to application to polyacylamide gels. This work was undertaken for the following reasons:

1) A rapid method of staining proteins is of value for quickly assessing the purity of different virus fractions during virus or protein purification. It was considered that, in the absence of immune serum of proven specificity for the RNP, analytical polyacrylamide gels were better guides to the progress of internal antigen purification.

2) A more sensitive staining method would more readily reveal minor protein components of the virus preparation.

3) The covalent binding of negatively charged dye molecules to positively charged amino acid residues in the viral polypeptides

would assist denaturation and solubilisation of the viral polypeptides at neutral or alkaline pH.

4) A more satisfactory alternative to bromophenol blue was sought as a reference marker for molecular weight determinations by the polyacrylamide gel method of Shapiro et al. (1967), as used by Weber and Osborn (1969) and E.A. Haslam (personal communication). Bromophenol blue is an unsatisfactory marker because it migrates as a broad band, it migrates more slowly than several polypeptides (e.g. those of molecular weight 17,000 or less) and it elutes from the gel during staining-destaining procedures. The latter problem was overcome by Weber and Osborn (1969) who measured the distance migrated by the bromophenol blue and the length of the gel before and after staining, and from these values estimated the position the reference dye would have had in the stained gels; this procedure is time consuming and could compound errors in measurement. Glucagon as a reference marker has several advantages over bromophenol blue; it has a higher mobility and smaller spread, and does not elute from the gel during staining and destaining procedures. However, glucagon stains poorly with amido black and Coomassie blue, and cannot be used as a reference marker in gels being stained for glycoprotein with the periodic acid-Schiff staining procedure. Glucagon prestained with Remazol blue or red has none of these disadvantages and was thus used as a reference marker in later work.

5) The intensity of staining of proteins with reactive dyes is proportional to the protein concentration (Fazekas de St.Groth et al.,

1963). Prestaining with Remazol blue (also a reactive dye) would thus permit a more accurate estimation of relative proportions of viral polypeptides (by scanning gels with a densitometer and integrating the area under the peaks) than would be possible with substantive dyes (e.g. amido black or Coomassie blue). As no densitometer was available, this has yet to be done.

6) Protein stained with Remazol blue may be used to monitor polyacrylamide gel systems and evaluate the method of recovering protein from the gels. Difficulty was encountered earlier (Chapter 5) in estimating recoveries of protein from gel slabs since the presence of bound SDS in recovered protein was not excluded. By measuring dyed protein spectroscopically, this difficulty may be overcome. Moreover, the efficacy of desalting procedures may be checked by correlating the optical density of the protein before and after with its dry weight. The preparative polyacrylamide gel technique, and recovery and desalting procedures, have been modified (Paper 6) and Remazol-dyed proteins have been used in their evolution and evaluation.

Paper 3 is concerned with the preparation of pure and highly infectious virus of the BEL strain. Although two cycles of adsorption-elution on human erythrocytes are adequate for preparing, in quantity, purified virus of a number of strains, for work on the protein chemistry of the virus, this procedure has a number of drawbacks (enumerated by Frommhagen and Knight, 1959), especially

for preparing virus of high infectivity. Cycles of adsorption-elution of BEL with human erythrocytes are not possible (fowl cells were not available) since extensive haemolysis results even on the third cycle. A variety of initial concentration procedures as alternatives to red cell adsorption-elution were tried, and batch adsorption-elution on aluminium phosphate proved the most satisfactory. No satisfactory alternative to centrifugation was found for concentrating eluates. The buoyant-density and rate-zonal techniques have been modified by including glycerol, which is known to preserve viral infectivity. SDS has been used to destroy preferentially non-infectious virions of the BEL strain, leaving a highly infectious preparation of virus. The use of the Remazol prestaining technique for monitoring viral purity is illustrated in Papers 2 and 3.

When a circulating pump became available, the polyacrylamide gel technique used in the previous work (Appendix 2) was abandoned in favor of the buffer system of Maizel (1966; but without urea) for a number of reasons:

1) The presence of glycine and urea in the buffers used in the previous work unnecessarily complicated experiments with the isolated polypeptides. The high buffering capacity of the tris-glycine buffer was no longer important when SDS-phosphate buffer in the electrode compartments could be recirculated.

2) The presence of a buffer discontinuity increased the risk of low molecular weight proteins "stacking" at, and moving with, the buffer

front.

3) By using gel systems used by other workers, results could be more directly compared.

4) The use of the SDS-phosphate buffer system permitted many estimates to be made on the molecular weight of the viral polypeptides.

In work described in Paper 5 it became apparent that the largest major polypeptide of BEL migrated more slowly when denatured without simultaneous reduction. To interpret this finding, it was necessary to first establish whether the rate of migration of polypeptides in SDS-polyacrylamide gels is affected by unfolding of the polypeptide following cleavage of disulphide bonds. The effect cystine bridges have on the mobility of polypeptides in 5% ~~polypeptides in 5%~~ polyacrylamide gels is described in Paper 4. This work had to be performed with proteins of known cystine content. The use of polypeptides linked with glutaraldehyde is described, and three conclusions were reached which were relevant to polyacrylamide gel analysis of influenza polypeptides. Firstly, failure to disrupt cystine bonds results in a significant increase in mobility in most cases; this finding might explain variations in the molecular weights ascribed to influenza virus polypeptides by different workers, since some have used for standard curves cystine cross-linked proteins of high molecular weight. Secondly, the mobility of the polypeptides relative to glucagon was remarkably constant from one experiment to another for most proteins, indicating that

the technique was reproducible and reliable, although it was shown that low molecular weight proteins did not always migrate at a rate proportional to the $\log_{10}$ of their molecular weight. Thirdly, the high temperature, high pH denaturation procedure employed appeared to give no polypeptide chain cleavage with the proteins used.

This paper also demonstrated that glutaraldehyde cross-linked proteins migrate as relatively compact polypeptide bands. A project was initiated to investigate (using glutaraldehyde) which polypeptides were in intimate contact in the intact virion, since only those polypeptides which are virtually touching may both simultaneously react with the same glutaraldehyde molecule, it was hoped that this work would provide evidence for some of the associations suggested in Paper 1, especially that between the RNP and the proposed 'core' antigen. However, completion of this project awaited the introduction of a denaturation procedure which could be shown to break down the virion proteins into the smallest units linked by covalent bonds other than cystine.

In Paper 5, the conversion of the virion into its smallest subunits was investigated. For this work, egg-grown virus was used for the following reasons:

1) Larger amounts of virus may be grown for a given expenditure in time and materials. Projected work on the protein chemistry of the virion requires large amounts of virus, which can only be satisfactorily produced in eggs.

2) Egg-grown virus generally contains a greater proportion of infectious virus than that grown in tissue culture. It is not practicable to label egg-grown virus with radioactive amino-acids or aminosugars, since this can only economically be done in de-embryonated eggs, which produce much incomplete virus.

3) It was considered that, due to differences in "host-antigen" content, disruption techniques applicable to egg-grown virus might give different results with tissue culture-grown virus. This has been found to be the case by others (E.A. Haslam, personal communication). In preliminary experiments, rapid qualitative analysis is more desirable than accurate quantitative analysis, and for this reason (and those indicated above) disruption of the virion was investigated using egg-grown virus stained with dyes for protein and carbohydrate.

The results presented in Paper 5 indicated that certain of the larger virion polypeptides were only partially converted into subunits, the extent to which this occurred depended on the denaturation procedure used. It was concluded that the only effective denaturation procedure unlikely to cause peptide bond cleavage is oxidation with performic acid followed by reduction in nonionic detergent ~~in~~ solutions saturated with guanidine hydrochloride and urea, followed by treatment with SDS and urea under reducing conditions. Virus treated in this way had a number of minor components of variable molecular weight, and two major polypeptides of quite low molecular weight, all of which appear to be derived from high molecular weight

glycoproteins.

The addendum is concluded with a discussion of all the work presented in this thesis. Those portions of the previous submission which may still be valid, but on which no additional work has been done, are indicated. Also indicated are, those portions which are no longer valid, those which may still be valid, and those which are definitely valid in the light of more recent work reported here, and by other workers elsewhere.

PAPER 1.

The fine structure of influenza virus.

INTRODUCTION.

Particles of non-filamentous strains of influenza virus are often pleomorphic, their largest dimension being 800 - 1800Å with an envelope 70 - 100Å thick from which arise spikes 90 - 100Å long, 15 - 20Å wide and 70 - 80Å apart; the envelope is a lipoprotein and encloses a coil of RNP (Hoyle, 1952; Horne et al., 1960; Hoyle et al., 1961). Two surface antigens of influenza virus have been separated and identified, namely the HA (Laver, 1964; Laver and Webster, 1968; Laver and Valentine, 1969) and the neuraminidase (Laver, 1963; Kilbourne et al., 1968; Laver and Valentine, 1969). The subunits investigated by these workers have been derived from influenza virus strains containing HA or neuraminidase resistant to the detergent sodium dodecyl sulphate (SDS). It has long been thought that the HA corresponded to the viral "spikes" described by Horne et al., (1960); Laver and Valentine (1969) confirmed this, and also showed that the neuraminidase resembled a 'mushroom' in shape, and postulated that this structure was present on the surface of the virion.

At present only a single internal antigen is generally recognised: the RNP. The quaternary structure of this RNP is variable, but is generally believed to be a protein-RNA complex, 50 - 70Å in diameter, coiled into a helix 600Å in diameter and of variable length (Hoyle et al., 1961; Waterson et al., 1963; Apostolov and Flewett, 1965). This RNP has also been purified from disrupted virus, but it then appears to be in fragments 500 - 1300Å in length and 75 - 150Å in width containing subunits 30 - 45Å in diameter

(Hoyle et al., 1961; Ruttkay-Nedecky et al., 1968; Pons et al., 1969).

This paper is largely concerned with the fine structure of the BEL and $X7F_1$ strains of influenza A, as revealed by partial disruption of the virion resulting from prolonged storage, or treatment with detergents. The origin of pleomorphic virus and the possible existence of a second internal antigen are discussed, and evidence is presented for the existence of the RNP helix in a variety of forms. Evidence is also presented suggesting that the haemagglutinin spike has a forked substructure, that the Ao and A2 neuraminidase have similar structure, and that some BEL virions are resistant to ionic detergents.

MATERIALS AND METHODS.

Detergents.

Sodium dodecyl sulphate (SDS), (obtained from Matheson, Coleman and Bell) was used as a 10% (w/v) solution. Sodium deoxycholate (DOC) was prepared by neutralisation of solid recrystallised deoxycholic acid with NaOH. Deoxycholic acid was prepared by acidifying a solution of DOC (reagent grade, British Drug Houses) and recrystallising the recovered precipitate from acetic acid-water. Tween 80, obtained from Atlas Powder Company, Wilmington, Delaware, was deionised as a 10% (v/v) solution by passage through a column of mixed bed resin.

Virus.

Virus was obtained from stocks held at -70° at the Australian National University. BEL virus seed had an ID_{50} of $10^{8.66}$ units/ml on thawing and ID_{50}/HA value of $10^{4.71}$. The Ao - A2 recombinent $X7F_1$ was that described by Kilbourne (Kilbourne et al., 1967). About 0.05 ml of a 1:1000 dilution of virus was inoculated into the allantoic cavity of 10 - 11 day-old fertile chicken eggs. Eggs were incubated at 36° for about 40 hours before chilling at -10° for one hour.

Purification of virus.

Infected allantoic fluid was filtered through a double layer of fine-mesh nylon cloth, chilled, and adsorbed with twice-washed fowl erythrocytes at 4° for about 45 minutes. The cells

recovered by centrifugation (900 g, 5 minutes) were washed once with ice-cold saline and suspended in 1/10 the allantoic fluid volume of Hanks BSS at 37° for 1 - 2 hours. Virus recovered by elution from these cells was clarified by centrifugation (2,000 g, 15 minutes) and concentrated by centrifugation (one hour, 19,000 revs/min in the Spinco 21 L rotor) onto a 0.40 ml sucrose cushion (60% in 0.15 M-NaCl). On completion of centrifugation, the loosely packed pellet and sucrose cushion were resuspended in a little of the supernate and subjected at 0° to ultrasonication (2 - 3 minutes, maximum output of a Mullard ultrasonic drill) followed by dialysis overnight against 50 - 100 volumes of 0.15 M-NaCl containing 2% (w/v) sucrose. The virus was resonicated, clarified by low speed centrifugation (4,000 g, 15 minutes) and layered on a 26 ml linear sucrose gradient (5 - 20% w/v sucrose in 0.15 M-NaCl buffered with 0.01M-tris-HCl, pH 7.2). Tubes were centrifuged for 40 minutes at 12,000 revs/min in the Spinco SW 25.1 rotor. The virus band was recovered from these gradients through a hole pierced at the bottom of the tube to one side of the pellet, and concentrated by centrifugation onto a sucrose cushion as described above. This virus, resuspended with sonication, was visibly contaminated with red cell debris. The latter was separated from the virus by centrifugation into a 25 ml preformed linear density gradient consisting of a mixture of sucrose and potassium tartrate (Griffith, 1968). A stock solution containing 26% (w/v) sucrose and 17% (w/v) potassium tartrate and 0.1% (w/v) sodium azide was used as the heavy component. The light component

was prepared by mixing three volumes of this with one volume of distilled water. Gradients were centrifuged for 90 minutes at 23,000 revs/min in a Spinco SW 25.1 rotor. The lower band containing the virus was recovered and stored for four weeks at 4°.

Preparation of virus for electron microscopy.

Virus was dialysed at 4° against 100 volumes of 0.15 M-NaCl, buffered at pH 7.2 with 0.01 M-tris-HCl, to remove sucrose and tartrate. BEL virus was treated with two equivalents (weight for weight) of sodium deoxycholate or sodium dodecyl sulphate at room temperature for 30 minutes. $X7F_1$ was sonicated (5 minutes, maximum output of a MSE ultrasonic drill) in the presence of two equivalents (weight for weight) of Tween 80 at 0°; the haemagglutinin titre was reduced by approximately 90%, but the neuraminidase activity was unaltered.

Virus treated with DOC was similarly dialysed, but the detergent formed a weak gel and was only slowly removed; thus dialysis was continued with one change for a further 24 hours.

Dialysed virus was diluted approximately tenfold with 0.15 M-ammonium acetate at room temperature. One drop of this material was placed on a carbon-coated electron microscope grid, followed by a drop of 4% (w/v) aqueous sodium silicotungstate. Excess fluid was drained off with the aid of filter-paper, and the grids air-dried. BEL virus preparations were examined with a Philips EM200 electron microscope, operated at 60kV and $X7F_1$ virus with a Siemens Elmiskope I microscope operated at 80kV.

Results and Discussion.

The origins of pleomorphic virus.

The BEL prepared as described contained much pleomorphic virus, but was seen to be largely free of non-virion material (Fig. 1). The reason for the presence of pleomorphic virus is not clear. One explanation may lie with the BEL seed used. This gave one ID_{50} per 60 - 100 virus particles on different occasions before purification; 90% of this infectivity was lost during purification (Griffith, in preparation+). The production of pleomorphic virus appears to occur most readily in cells in an abnormal physiological state, e.g. those treated with vitamin A (Blough, 1964a), or those present in de-embryonated eggs (Hanig and Steim, 1960). As one may regard an infected cell as becoming increasingly abnormal as infection proceeds, the production of pleomorphic virus late in infection may be a "normal" occurrence (von Magnus, 1957), especially in strains which grow to a high titre. In the extreme, with certain strains, this may take the form of filaments, as found by Ada et al. (1958); they showed that the Ryan F strain produced spherical virus during the first 21 hours after infection, and subsequently both short and long filaments. Short filaments were occasionally found in this preparation of BEL (arrow 'a', Fig. 1). Diploid and triploid forms of parainfluenza particles exist (Hosaka et al., 1966) and it has been proposed that these arise through random incorporation of genomes into virions during budding of virus from the cell surface (Dahlberg and Simon, 1969).

+Chapter 3

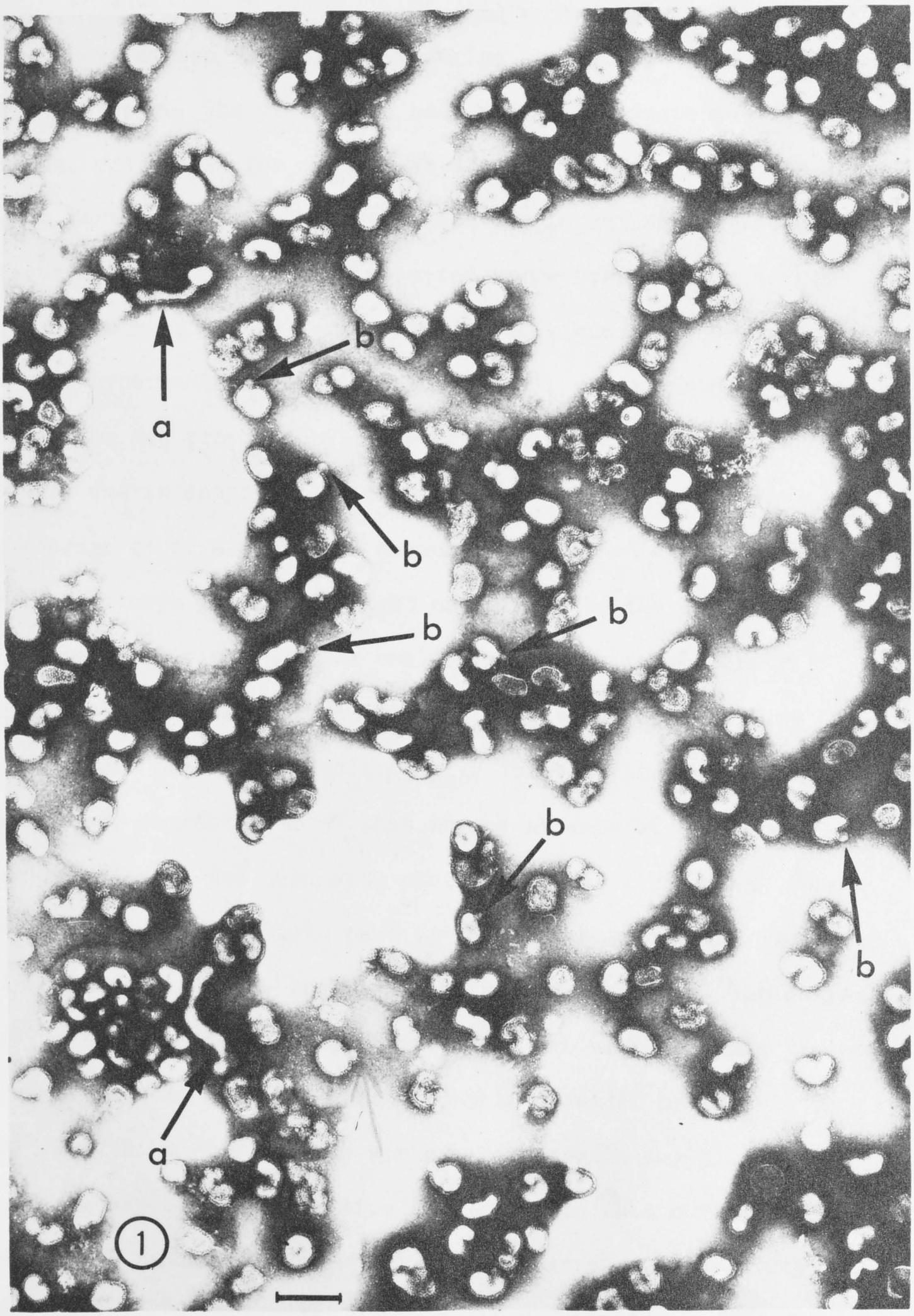


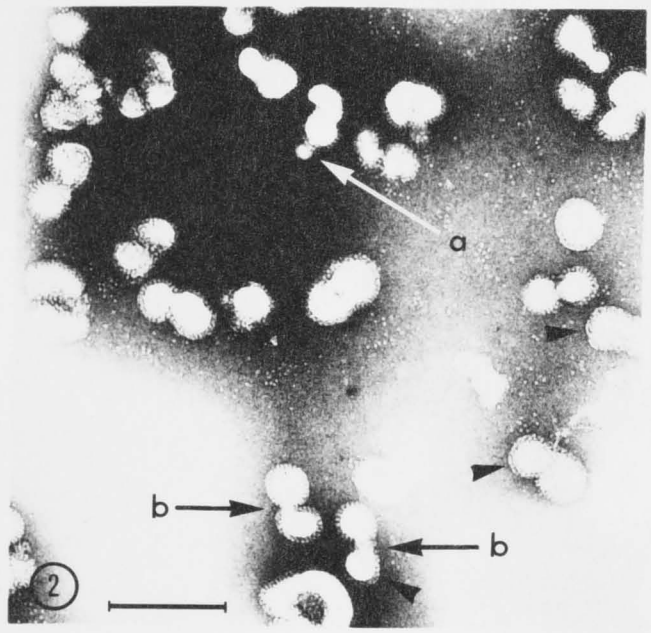
Fig. 1. The BEL strain of influenza virus; in addition to many pleomorphic virions, two short filaments may be seen ('a' arrows). Some particles show 'tongues' which have few or no HA spikes ('b' arrows). Scale line = 2,000 Å.

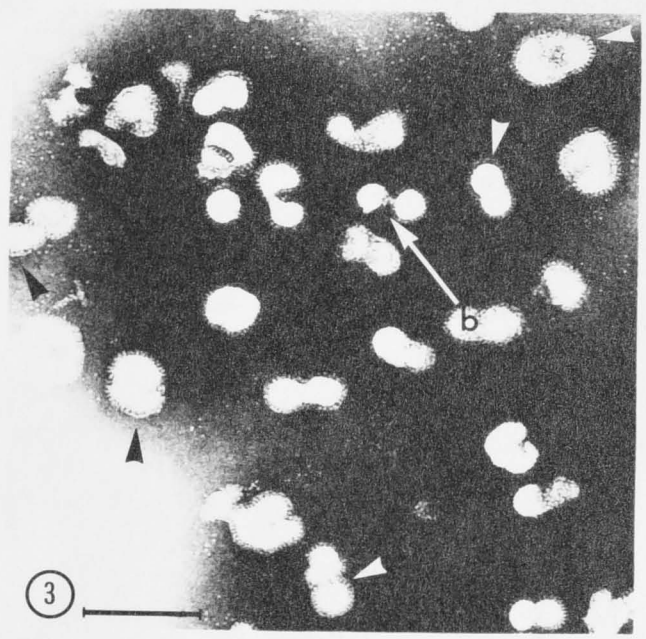
In addition to being formed during maturation, pleomorphic virus may have arisen in other ways during purification and subsequent storage. The virus used here had been resuspended with sonication, and stored for a month at 4° in a high concentration of potassium tartrate. This may have caused the formation of pleomorphic virus, since such virus is reported to be formed on "moderate sonication" (Simpson, 1964) or on storage of virus at 4° or in the presence of hypertonic saline (Blough, 1964b); no mechanism for this transformation was proposed by these workers. Some pleomorphism is undoubtedly due to dehydration of particles, perhaps by osmosis during storage or by evaporation during staining and drying on the electron microscope grid; this might especially be the case with viral filaments (Hoyle, 1954) or smaller particles incompletely filled with RNP. However, the ability of influenza virus to fuse with cytoplasmic membranes (Hoyle et al., 1962; Hoyle, 1962; Morgan and Rose, 1968) suggested to us that in the absence of available cell membrane (as is the case with purified virus) virions may fuse with each other. This would in part explain the heteroploid virus commonly observed in myxo- and paramyxovirus preparations. Sonication, 'ageing' or exposure to high salt concentrations may help this process by removing absorbed material (such as nucleic acids, or inhibitory mucoids) from the surface of the virion. The process may also be helped by high-speed centrifugation of virus onto tube surfaces, a procedure which is accompanied by significant loss of infectivity with influenza virus (Griffith, unpublished observations), as well as

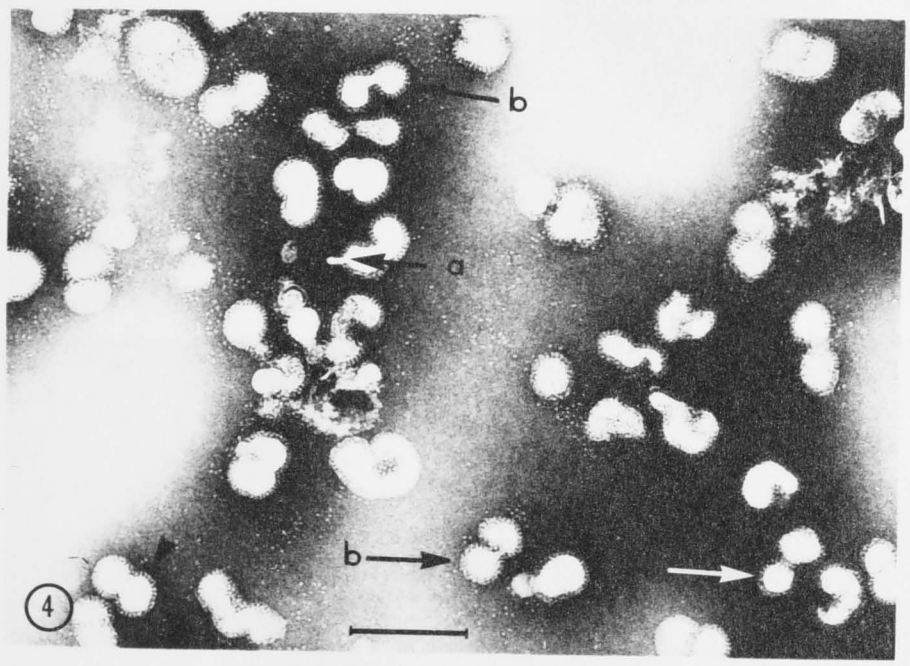
with a number of other viruses (Anderson and Cline, 1967). Spherical particles were frequently found in pairs (Fig. 2 - 5). These pairs may be particles in various stages of fusion, as is well shown in Fig. 6. If fusion does occur, one may predict that with increasing time of storage, the proportion of monoploid virus will decrease, while that of heteroploid virus will increase. This would be difficult to demonstrate experimentally by absolute particle counts at well spaced intervals, since particles have a tendency to disintegrate spontaneously during storage. Particles were often seen to contain what appeared to be 'tongues' of membrane (Figs. 1b, 2b, 4b) sometimes containing only a few spikes (Fig. 12d) or having a double membrane appearance (Fig. 13c). This could be the point of last contact between the virion and the cell membrane during virus release from infected cells. It may be the point of contact of the fusing virions, and the point of irreversible first contact during the viral invasion of uninfected cells.

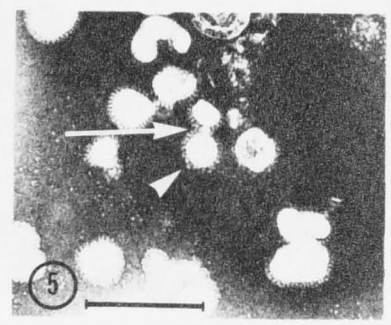
The lipoprotein shell of the virion.

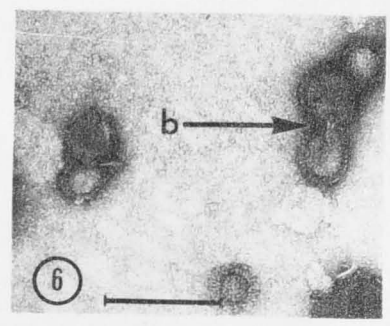
The virus preparation contained many particles penetrated by negative stain. At high magnification, an area free of stain and of width varying from 40 - 130Å was seen below the surface spikes of many of these particles (arrow "a", Fig. 7). In one particle, it was evident that the haemagglutinin spikes (150 Å in length) were embedded in this layer to a depth of 60 - 80Å (Fig. 7b). This particle also showed elements of a second layer beneath the outer membrane. Occasionally, this stain-free area was resolved as a double layer











Figs. 2 - 6. 'Tongues' were clearly visible on some particles of the BEL strain ('a' arrows). Particles were often seen in pairs. The outer shell of the virions at the point of contact were poorly penetrated by negative stain in several cases ('b' arrows). One structure was visible which might represent two particles in the process of fusing (Fig. 6). Structures which might be neuramidase molecules are indicated by arrow heads. Scale line = 2,000 Å.

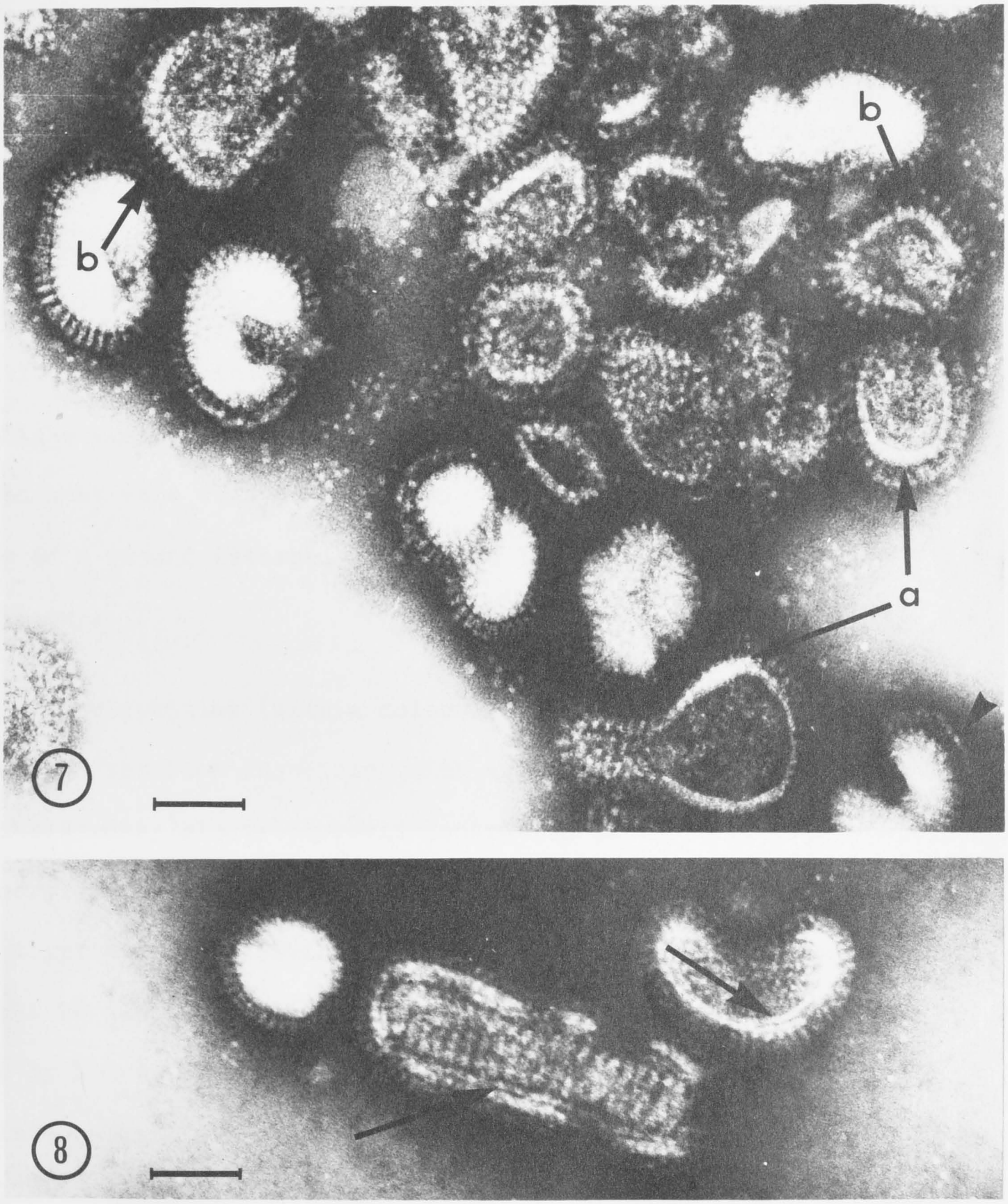


Fig. 7. Several virions of the BEL strain were penetrated by negative stain and showed an area free of stain below the surface spikes ('a' arrows). The spikes were occasionally seen to be embedded in this layer ('b' arrows). The arrow head indicated what may be a neuraminidase molecule. Scale line = 500 Å.

Fig. 8. Two particles showing the double layered structure of the outer shell of the virion (inner component arrowed). The RNP coil is clearly separated from this component in the largest particle. Scale line = 500 Å.

which was clearly not part of the RNP coil (arrowed, Fig. 8). The outer of these two layers is probably the lipid in which the spikes are embedded (Laver and Valentine, 1969) while the layer between this and the RNP coil is possibly a second internal antigen, and may correspond to the "inner leaflet" described by Compans and Dimmock (1969), or the "granular layer" of Apostolov et al., (1969), or the "inner apposition" of Bächi et al., (1969). To account for multiple precipitin lines obtained on immunodiffusion of "soluble" antigen preparations against specific antisera, Hana and Hoyle (1966) suggested that more than one antigenic form of the RNP existed; the presence of a second internal antigen would, in part, also explain their findings.

A polypeptide (with a molecular weight of about 23,000) which has not yet been associated with any of the known viral antigens may be isolated by electrophoretic separation of the virion polypeptides in SDS-acrylamide gels; many neutral peptides are found in peptide maps of tryptic digests of this polypeptide (Griffith, in preparation[+]). This might be expected of a protein whose function is to stabilise a membrane by hydrophobic interaction with the membrane lipid. If a second internal antigen distinct from the RNP does exist and is lipophilic in character, one might expect it to react with polar lipid or detergents without being denatured or disorganised. The remarkable resistance of some influenza virions to disruption by ionic detergents (Webster and Laver, 1966) may thus be explained in part by the stabilising effect this protein might have on the lipoprotein shell

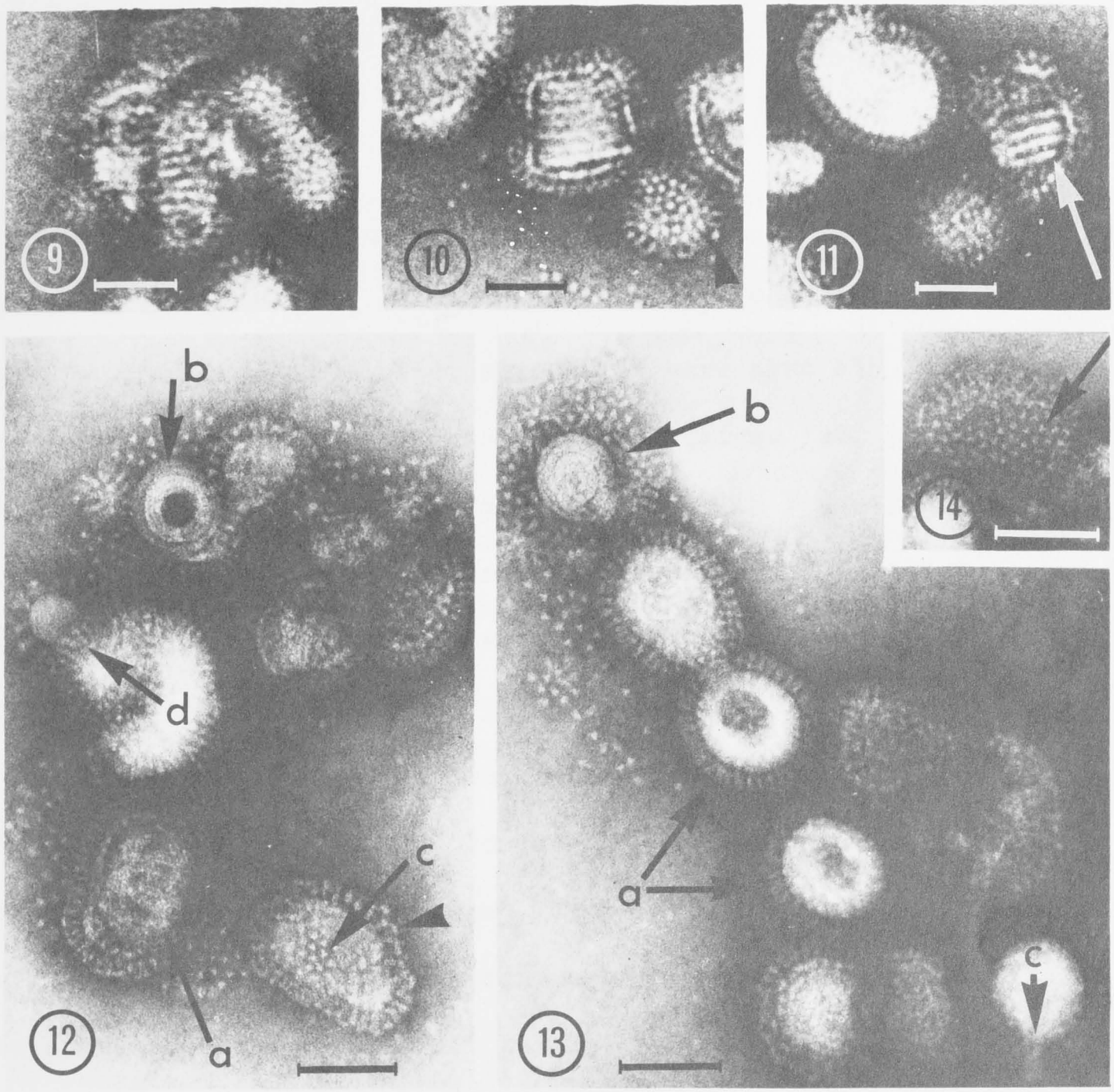
[+]Chapter 2, Chapter 5

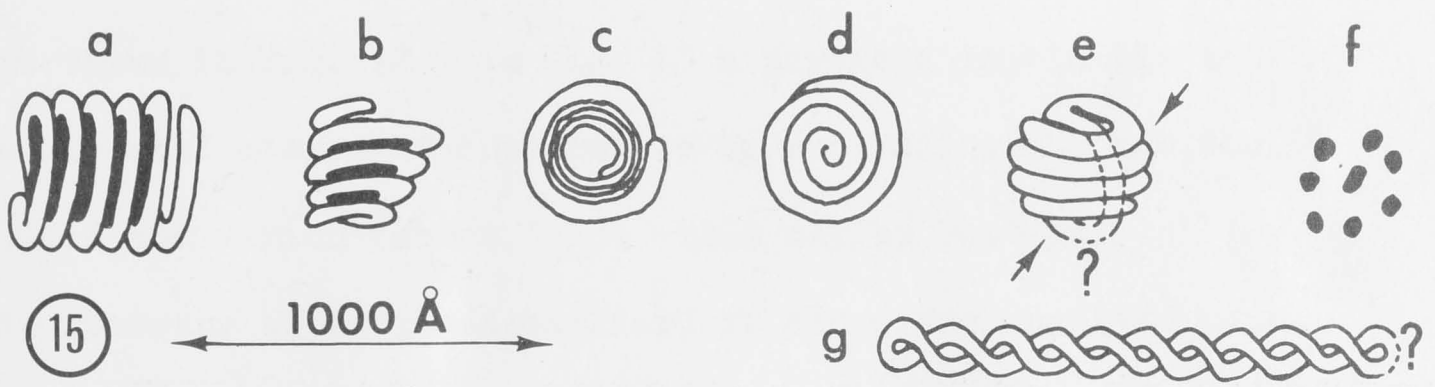
of the virion. We may further speculate that this protein assists assembly of the virion by simultaneously recognising and interacting with the hydrophobic foot of the haemagglutinin spike, as well as the membrane lipid and the RNP coil. Alternatively, this protein may play a role during infection of cells by assisting the dissolution of the cytoplasmic membrane which Morgan and Rose (1968) believe accompanies viral invasion of the cell.

The structure of the ribonucleoprotein.

The RNP coil shown in Fig. 8 appears to consist of a helix of 19 or 20 turns. Other particles with a smaller number of turns to the helix were also found (Figs. 9, 10, 11); in one case, the diameter of the helix decreased at the lower end (arrowed, Fig. 11). The diameter of the coil varied from 350 - 600Å, while each turn was 50 - 65Å thick and separated by a gap 25 - 40Å wide. These particles show parallel bar structures which were interpreted by Hoyle et al., (1961) as a parallel arrangement of RNP fragments. It is now more generally believed that the RNP is present as a helix in the form of an extension spring (Waterson et al., 1962; Apostolov and Flewett, 1965). Apostolov and Flewett reported particles containing 6, 12 or 18 turns to the helix, and postulated that a basic RNP unit containing 6 turns was normally present, and that the other particles were "diploid" or "triploid" states. In our virus preparation, this was not the rule.

Occasionally, particles apparently containing disorganised and loosely coiled RNP were also seen. Such a particle is shown in





Figs. 9 - 14. The various forms of the RNP coil are clearly seen in these figures. The coil has variable dimensions; in one case, a side-view shows the coil decreasing in diameter at its lower end (arrow, Fig. 11). From above, the coil appears as a spiral coil, both complete (Fig. 13, 'b') and incomplete (Fig. 12, 'b'). Intact particles which probably contain incomplete spirals are shown in Fig. 13 ('a' arrow). Elements of a disorganised RNP coil may be seen in Fig. 12 ('a' arrow). Tongues of virion-associated material are shown in Figs. 12 ('d' arrow) and 13 ('c' arrow). Arrow heads indicate structures which may be neuraminidase molecules. Fig. 12 ('c' arrow) and Fig. 14 show an end-on view of the spikes; evidence of possible trimeric structure is seen in two of these spikes. Scale line = 500 Å.

Fig. 15. This shows a schematic interpretation of the various forms of the RNP (for details, see text).

Fig. 12(a).

RNP spirals resembling watch springs have been described by others (Horne et al., 1960; de Thé and O'Connor, 1966; Ruttkay-Nedecký et al., 1968). Some similar structures were also clearly seen where particles had partially disintegrated (Figs. 12b, 13b). These coils were 350 - 450Å in diameter; that shown in Fig. 13 had a strand width of about 50Å. One of these spiral coils had a hollow centre (Fig. 12); presumably it represents an end-on view of a structure of the type shown in Fig. 11. The outer layer of the coil shown in Fig. 12 appeared to differ in staining properties from the rest of the RNP. Perhaps this was because the outer layer is a part of the proposed second internal antigen, or perhaps because it represents a single thickness of RNP, the centre of the coil consisting of several superimposed strands. Intact particles with dark centres were also present (Fig. 13a) and these presumably contain RNP coils of the type shown in Fig. 12. In Fig. 13 a particle is visible in which some internal component appeared to be protruding through the outer shell of the virion (arrow 'c'). This may be residual cytoplasmic membrane as proposed earlier, or since each had the same width (about 50Å) as the coiled RNP strand (arrow 'b'), it may be two strands of RNP.

It is evident from our results, and those of others, that the RNP may be arranged within the virion in a number of ways, and these are indicated diagramatically in Fig. 15 (a-d). No structures were seen which might correspond to a side-view of the complete

spiral coil, and we can only speculate on its appearance. The Takátsy loop model shown in Fig. 15e is one possibility. This is similar to the model proposed by Horne and Wildy (1961) or the "ball-of-string" model of Blough (1964a). However, to account for the observations of Compans and Dimmock (1969), we suggest that a strand of RNP passes through the middle as shown. A thin cross-section of this model, at the points indicated by arrows, would have the appearance indicated in Fig. 15f. Such an arrangement of dark spots (including the central one), each 50Å in diameter, is common in thin sections of influenza particles (Compans and Dimmock, 1969).

Influenza virus RNP can only be extraced in several short fragments of varying length (Duesberg, 1969; Pons et al., 1969; Kingsbury and Webster, 1969). Furthermore, long-lived, high molecular weight RNA (Duesberg and Robinson, 1967; Pons and Hirst, 1968) or RNP (Duesberg, 1969) precursors of these fragments cannot be found within infected cells. The appearance of the RNP in disintegrating particles as a coil without any obvious breaks is difficult to reconcile with these findings. It has been suggested that the RNP fragments are linked end-to-end by weak secondary forces readily broken mechanically, (Pons et al., 1969). This could explain the failure to observe intact coils (other than in particles penetrated by stain) after shaking virus with ether (Hoyle et al., 1961; Ruttkay-Nedecký et al., 1968) or treating virus with detergents (Duesberg, 1969; Pons et al., 1969; Kingsbury and Webster, 1969). However, as pointed out by Almeida and Waterson (1969), we cannot be certain that some of the

RNA (and hence RNP) is not cleaved into fragments by hydrodynamic forces during isolation, as is the case under certain circumstances with Turnip yellow mosaic virus (Matthews and Ralph, 1966) and Cowpea chlorotic mottle virus (Bancroft et al., 1968). The RNA in influenza virus RNP may be cleaved by ribonuclease, and it is possible that some of the influenza virus RNA is fragmented by contaminating nucleases, as is found with the RNA of Cucumber mosaic virus (Francki, 1968) and Alfalfa mosaic virus (Boll and Veldstra, 1969).

We favour an alternative explanation for the failure to find RNP in lengths of several 1000 Å. In the preparation of RNP described by Pons et al. (1969) may be seen a structure 150 Å wide and nearly 2,000 Å long, containing a helical mid-section and terminal loops. If the RNP coil shown in Fig. 15e had free ends, then on its release from the virion it might be expected to uncoil giving a single strand several 1000 Å long and 50 Å wide. If it was present as a loop of RNP this could not occur, but rearrangement of the coil, perhaps with some tightening, might give the structure shown in Fig. 15g, which depicts diagrammatically the structures shown by Pons et al.(1969). The 2,000 Å structure of these workers would thus be a single RNP strand (considerably longer than 4,000 Å) in the form of a loop which, perhaps because of some internal stress, had twisted into a rope-like structure, which would be at least twice the width of the strands seen in the RNP coils of collapsing particles. This continuous loop model is consistent with the suggestion (Mackenzie, 1968) that the RNA of influenza virus behaves in the infected

cell as a circular functional unit.

We propose that the influenza virus RNP is incorporated into the spherical particle as a continuous loop of variable length, but probably 8,000-10,000 Å long(FrischNiggemeyer,1961), coiled into a Takátsy loop-like structure.

It is not known what fills the interior of these RNP coils. The cavity might be filled with fluid, or a matrix of RNP and lipid (Kates et al., 1962) or a haemagglutinin gel (Hoyle, 1968). Alternatively passively incorporated cell cytoplasm might be present (Hoyle, 1954), perhaps containing nucleases; the presence of cell material is suggested by the work of Ada and Perry (1958) who showed that host-cell RNA is incorporated into viral filaments. It is also possible that the RNP coil is embedded in the proposed second internal antigen. Two viral polypeptides are found initially in the nucleus of infected cells (Taylor et al., 1969). The larger of these (molecular weight 50,000) is derived from the protein of the RNP (White et al, 1970) and the smaller (molecular weight 20,000) contains as much as 40% of the radioactive amino-acids incorporated into egg-grown virus (E.A. Haslam, personal communication). As suggested earlier this smaller unidentified polypeptide may be part of a second internal antigen, and it is possible that the virion is filled with a core (which is assembled in the nucleus of infected cells) consisting of RNP embedded in a matrix of this internal antigen. The susceptibility of purified influenza RNP to ribonuclease (Hoyle, 1952;

Schäfer and Zillig, 1954; Duesberg, 1969; Pons et al., 1969) suggests that the RNA of the RNP is exposed. The presence of many basic peptides (as well as neutral peptides) in tryptic digests of this polypeptide (Griffith, in preparation) is consistent with a protective interaction between the basic residues of this protein and the acidic phosphate groups of the exposed RNA. We suggest that this postulated second internal antigen be called the 'core' or 'packing' antigen.

The HA and RNP of DOC-disrupted virus.

The naked coils shown in Figs. 12 and 13 were surrounded by groups of short rods, which resemble the viral HA isolated by Laver and Valentine (1969). In addition, many triangular particles with a side of about 45 Å were visible scattered over the background. Since the triangles could also be seen in intact particles (Figs. 12c, 14), it was thought likely that they represented the HA spikes viewed end-on. Triangular structures have been reported previously (Rowe and Williams, cited by Cruickshank, 1964), and were thought to be neuraminidase. On the basis of the work of Laver and Valentine (1969), and that reported here, it is more likely that these were in fact HA monomers mounted vertically on the grid. The reason for their vertical stance is presumably a result of the hydrophobic foot of the molecule (postulated by Laver and Valentine, 1969) interacting with the hydrophobic carbon on the grid. It was thought that in the presence of an ionic detergent this interaction might be prevented. Consequently, virus was treated with DOC, and mounted on the grid in

Fig. 16. The BEL strain, treated with DOC, is seen to be reduced to fragments of varying size. Rod-like HA subunits ('a' arrows) and mushroom-like subunits ('d' arrows) have been released from the virions. Fragments of what is probably RNP may also be seen ('c' arrows). The shorter HA monomers sometimes seem to be forked at one end ('a' arrows). The end-on view of the HA spikes again suggests a trimeric subunit structure ('b' arrows). The neuraminidase molecules, alone or grouped with other subunits, are frequently seen ('d' arrows). Their dimensions vary depending on how obliquely the molecules are being viewed (see Fig. 24). Scale line = 1,000 Å

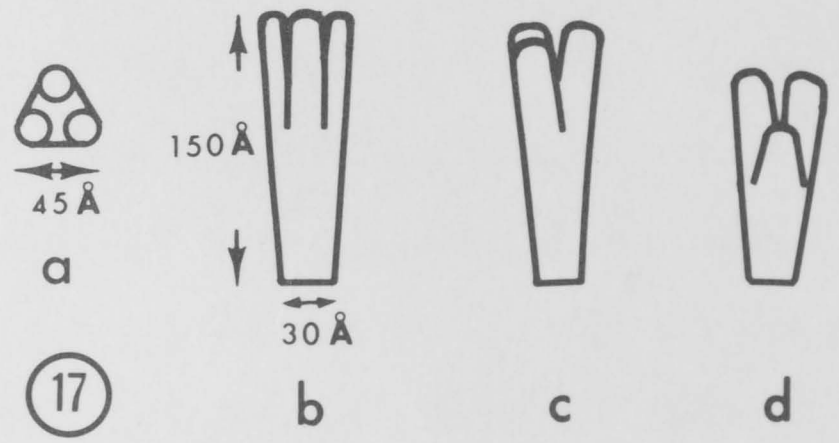
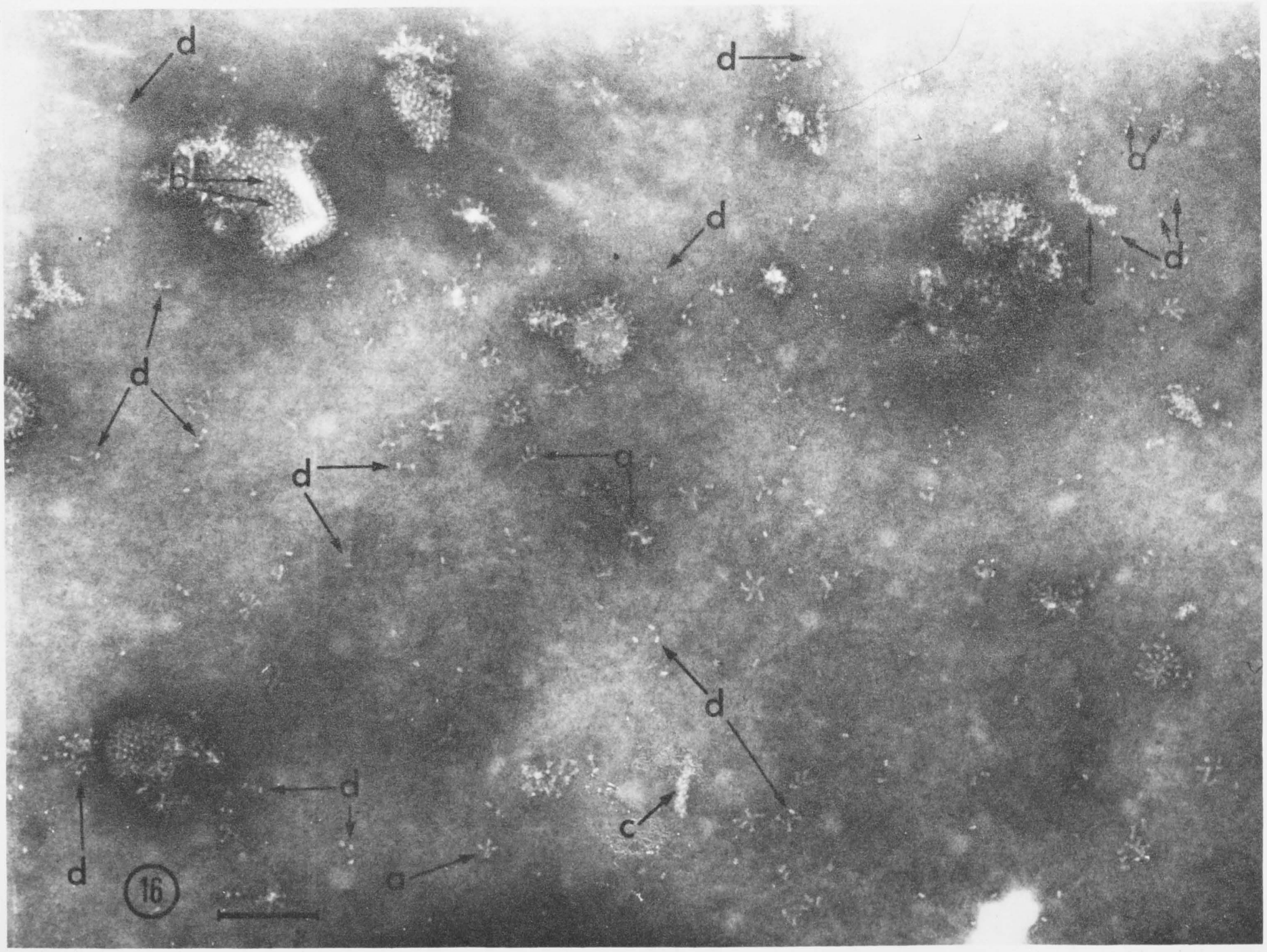


Fig. 17. Schematic interpretation of the HA sub-structure. (a) end-on view of the HA spike. (b) side-view of the HA spike. (c),(d) two views of the HA spike, demonstrating that oblique views result in foreshortening which permits the forked nature of the HA spike to be seen.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16
a

the presence of traces of this detergent. The number of individual monomers found standing vertically was much reduced (Fig. 16) although groups (or "rosettes") were still present. Many of the rods were wider at one end and sometimes seemed forked (Fig. 16a). The triangular end-on view of these rods suggested that this forking may be three-pronged. Such a structure was seen in end-on views of the HA spikes in virions both before (Fig.12c, 14) and after DOC-treatment (Fig. 16b). The detached spike was about 30 Å wide at the base, but broadened to 40-50 Å towards its forked end. The length of the HA monomers appeared to vary from 80 Å to 150 Å; presumably the shorter rods were not lying flat on the grid and thus appeared foreshortened. Our interpretation of the fine structure of the HA monomer is shown in Fig. 17. This figure shows how foreshortening may affect monomer length, and how the forking (if it exists) could be obscured, depending on the angle from which the rod is viewed.

A molecular weight of 150,000 has been proposed for the HA monomer (Laver and Valentine, 1969). Separation of virion polypeptides (after reaction with performic acid) by electrophoresis in SDS-polyacrylamide gels reveals none with a molecular weight of this order; in fact the largest major polypeptide present, which has a molecular weight of about 60,000, is a glycoprotein and has been equated with the viral HA (Griffith, 1968, 1969). In making these estimates the presence of carbohydrate was not taken into account, and so the values are probably rather inaccurate, but they do indicate that the HA monomer very probably has a subunit structure.

Material resembling the RNP fragments of Hoyle et al. (1961) may also be seen in Fig. 16 (arrow c). In these there is little evidence of the secondary coiling demonstrated in RNP fragments by others (Ruttgay-Neckedy et al., 1968; Pons et al., 1969). This RNP is considerably wider than that present in the larger coils described earlier, being 110-130 Å in width. The two fragments shown were about 600 Å long; they may represent shorter and more tightly coiled structures than that depicted diagrammatically in Fig. 15g.

The neuraminidase of influenza virus.

In addition to fragments of RNP and HA, the DOC-treated BEL preparations also contained 'mushroom'-shaped structures resembling the neuraminidase of Asian (A2) influenza isolated from the recombinant $X7F_1$ and described by Laver and Valentine (1969), although the 'stem' and 'foot' of many were barely visible. These structures are present alone, or grouped with other subunits (Fig. 16d). The dimensions of the 'head' varied in both length (70-90 Å) and width (35-65 Å), while the stem-plus-foot appeared of variable length (60-120 Å) presumably because of foreshortening in those subunits viewed obliquely.

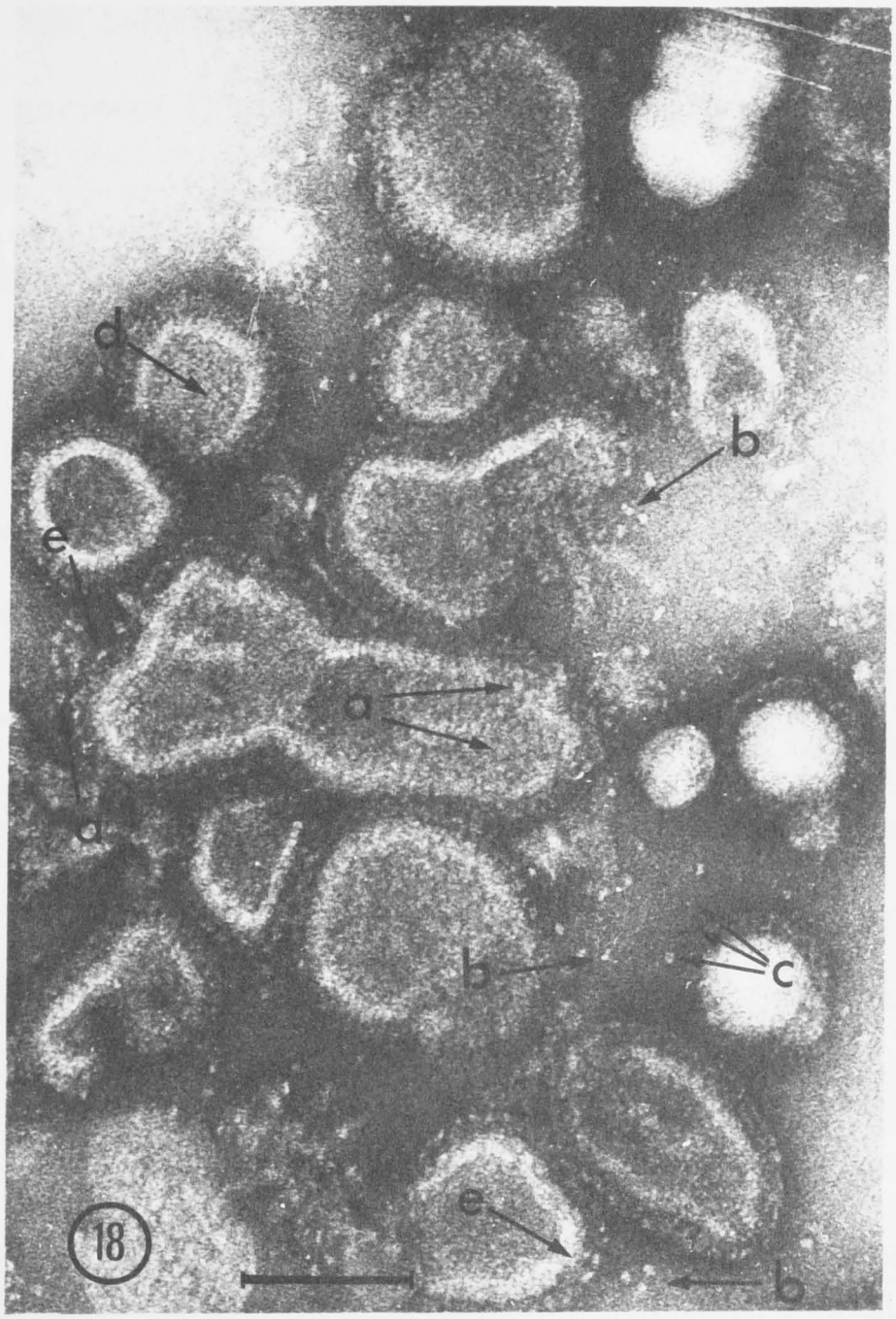
On occasions side-on views of the neuraminidase suggested that the head was composed of two spheres each 30-35 Å in diameter, while oblique views suggested a more complex sub-structure (Fig.16d) which in one case (top centre, arrow d, Fig. 16) appeared to be tetrameric.

Since the HA and neuraminidase of BEL and X-7FI differ chemically in their resistance to SDS, it was wondered whether they differed morphologically also. Even though the HA of X-7FI had been largely destroyed by sonication with Tween 80, structures were seen resembling the HA spikes of BEL, both in end-on and side-on projection (Figs. 18a, 19a), and they appeared to have similar dimensions to those of the BEL strain. Subunits resembling the neuraminidase isolated by Laver and Valentine (1969) from the X-7FI recombinant were also readily recognized in preparations of this virus. Some clearly had a head consisting of two spheres, while others, viewed obliquely, showed heads of more complex substructure. (Fig. 18b.). Also present were structures which might represent the foot and stem and head of the neuraminidase detached from each other. (Figs. 18c, 20a). In Fig. 18 the stem had a length of about 110 Å and the foot a diameter of about 20 Å. What may have been the detached head in one case appeared to have elements of a tetrameric substructure (Fig. 18c). The loss of its supporting stem may have resulted in the subunits moving closer to each other, since the subunits of two similar tetramers present on the surface of an intact virion (Fig. 18d) were more widely spaced. The dimensions of these two tetramers were 70-75 Å along the side, and about 85 Å across the diagonal; the subunits had a diameter of 30-35 Å. Fig. 24 shows a schematic interpretation of the fine structure of the neuraminidase molecule of the influenza virus as revealed in Figs. 16, 18 - 20. The effect foreshortening might have on the appearance and dimensions of the head and

Figs. 18 - 20. Virions of the X7F$_1$ recombinant after sonication with Tween 80. Some particles still have intact HA spikes which are seen end-on in Fig. 18 ('a' arrow) or side-on in Fig. 19 ('a' arrow). Neuraminidase molecules may be seen alone or in groups ('b' arrows, Figs. 18, 19); the complex subunit structure of the neuraminidase head can be seen in these molecules. Occasionally the head of the molecule is detatched from the stem and foot (Fig. 18,'c'; Fig. 20,'a'). Two structures with a tetrameric subunit could be distinguished in one virion in Fig. 18 ('d' arrow). Neuraminidase molecules were present as surface spikes in some particles (Fig. 18, 'e' arrow; Fig. 20, 'b' arrow).
Scale line = 1,000 Å.

Figs. 21 - 23. Untreated virions of the X7F$_1$ recombinant showing neuraminidase molecules in the surface of the virion. On occasions, fringes are seen which could be several enzyme subunits side-by-side. Scale line = 1,000 Å.

Fig. 24. Schematic interpretation of the fine structure of the influenza virus neuraminidase.
(a) Top view of the head of the molecule. (b),(c) Side views of the enzyme subunit showing how the length of the head may vary. (d),(e) Oblique views of the molecule showing how the width of the head and the length of the stem may vary. (f) Two neuraminidase molecules with stems overlapping.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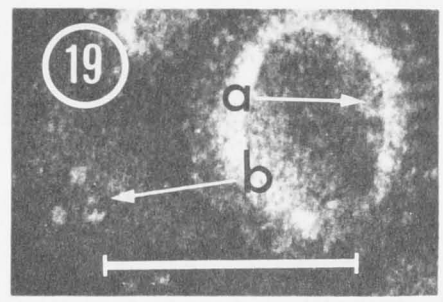
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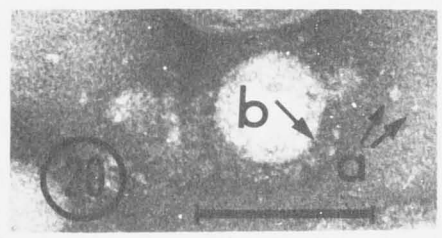
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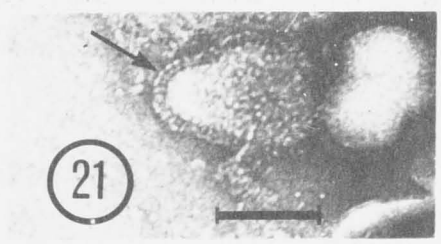
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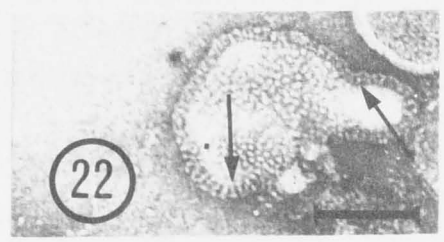
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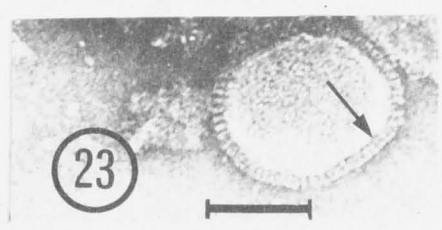
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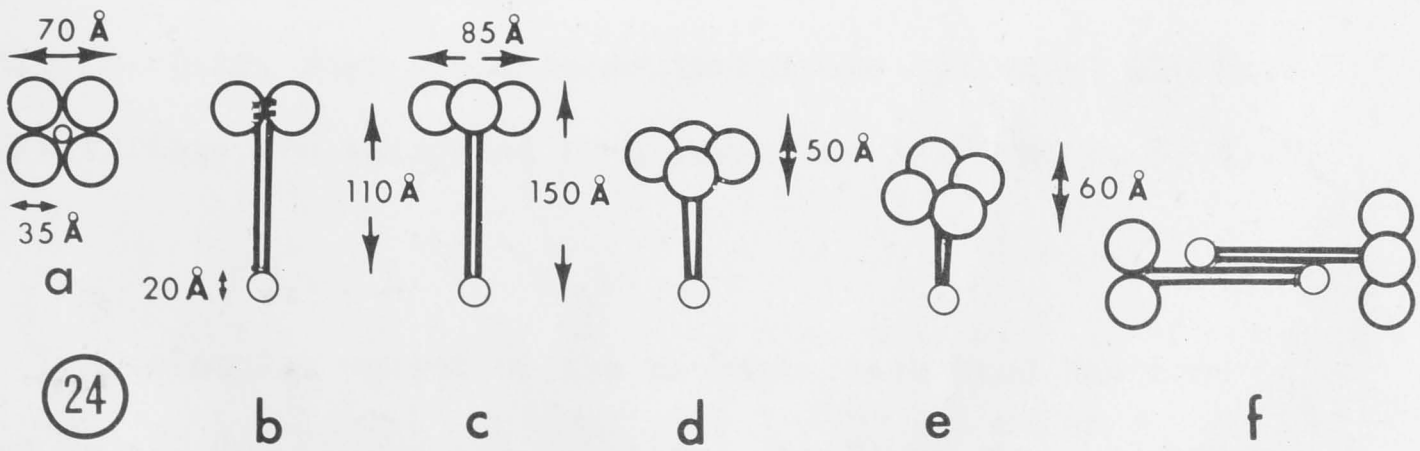

70 Å
35 Å
a
20 Å
110 Å
85 Å
150 Å
50 Å
60 Å
24
b
c
d
e
f

stem are clearly illustrated in Fig. 24.

The length of the neuraminidase molecule is similar to that of the HA monomer (about 150 Å). This suggests that the foot and part of the stem of the neuraminidase molecule are embedded in lipid to the same depth as the HA spike, i.e. 60-80 Å (Fig. 7). Thus at least a portion of the stem is likely to be lipophilic, and an attraction between the stems of two neuraminidase molecules would account for the presence of what appear to be pairs of these subunits with stems overlapping (Fig. 16d), as shown diagrammatically in Fig. 24f.

The head of the neuraminidase and HA molecule may often be distinguished by their different staining properties. Thus spikes of intact particles were often seen which were probably side-on views of the neuraminidase (Fig. 18e, 20b, 21, 22, 23). Where many neuraminidase subunits were present side-by-side, particles had a 'fringed' appearance, especially where the surface of the virion had little curvature. (Figs. 22, 23); it is clear that in these particles the 'crown' of the neuraminidase head projects to the same extent as that of the HA spike. What could be neuraminidase spikes at the surface of BEL virions are indicated by arrow-heads in Figs. 2, 3, 4, 5, 10, 12.

The molecular weight of the neuraminidase head has been calculated as 130,000 (Laver and Valentine, 1969) on the assumption it is a cylinder 85 Å long and 50 Å in diameter. The failure by these workers to resolve the fine structure of the head may be related to

their method of isolation of the molecule in the presence of SDS, which is accompanied by considerable losses of enzymic activity (Griffith, unpublished observations)+, and perhaps the detergent caused some disorganisation of the head of the molecule. The molecular weight of a sphere of protein (partial specific volume 0.73 cm^3/g.) 35 Å in diameter is close to 16,000. A tetramer of these subunits would give the head a molecular weight of 64,000. This is in good agreement with the value of 60,000 assigned to the major polypeptide believed to be derived from the neuraminidase (E.A. Haslam, personal communication). The sedimentation coefficient of purified influenza A_2 neuraminidase obtained by Rafelson et al., (1966) was 4.25. This indicates that the molecular weight of the neuraminidase is probably a little less than that of bovine serum albumin (67,000), which has a sedimentation coefficient of 4.3 (Tanford, 1961). It is not clear whether Rafelson et al.(1966) had isolated just the head of the neuraminidase; our results and those of E.A. Haslam suggest that this was likely. The presence of the stem and foot in the molecule may explain the higher S values obtained by other workers. (Noll et al. 1962; Laver 1963; Laver and Valentine, 1969). Whether the head is a single molecule or consists of subunits is not yet known. The ease of separation of the head and stem of the molecule demonstrated in Fig. 18 suggests that the head and stem are probably not joined by covalent bonds, although the possibility that traces of contaminating proteases in the virus preparation had separated the head from the stem cannot be excluded.

+Chapter 4

Structures resembling the neuraminidase of Drzeniek et al. (1968) were not seen. The structures reported by these workers may in fact be aggregates of RNP subunits, since they closely resemble rings present in the RNP preparations of Hoyle et al., (1961), Ruttkay-Nedecký et al., (1968) and Pons et al., (1969) both in appearance and dimensions.

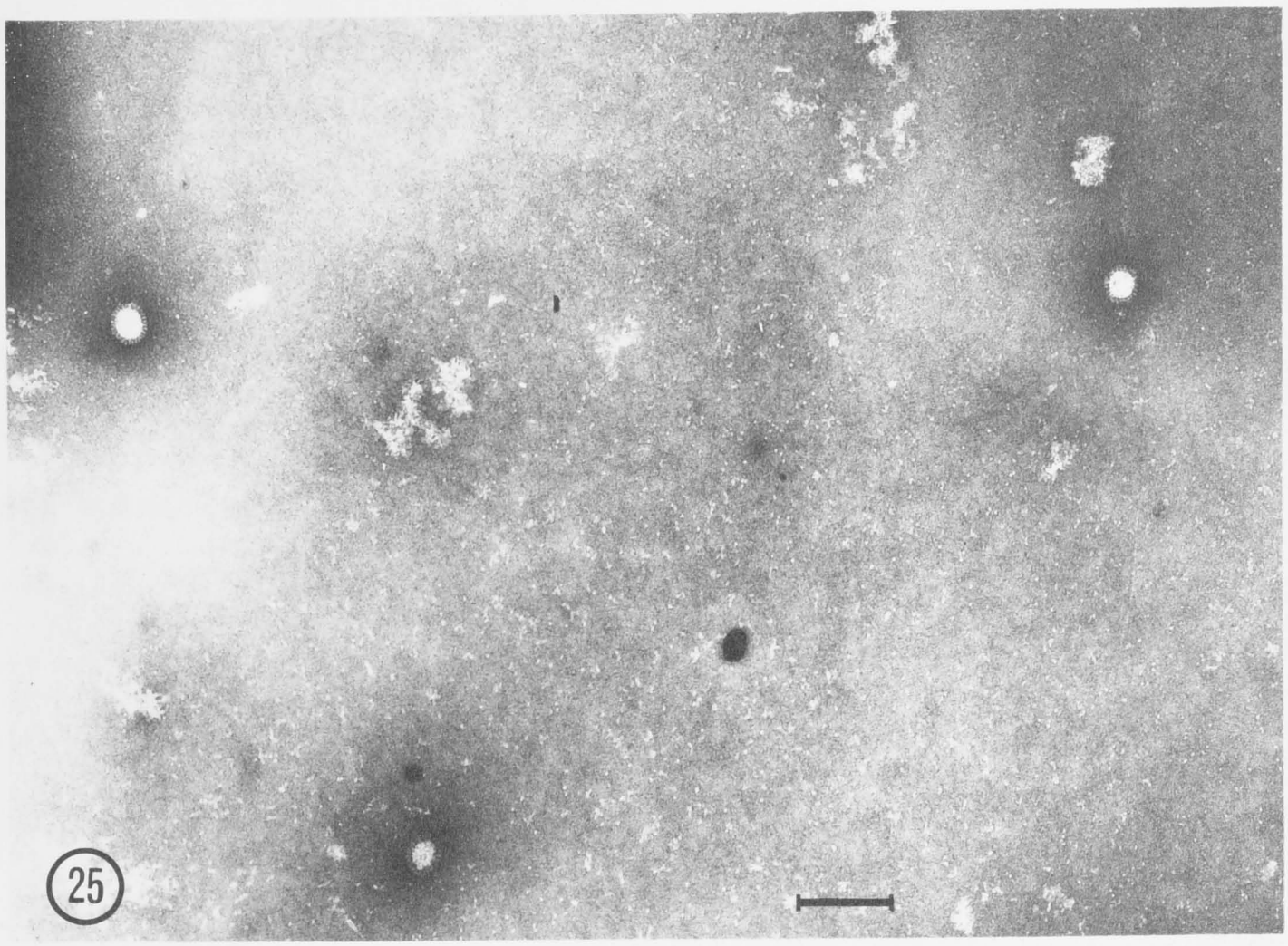
The effect of DOC and SDS on virion integrity.

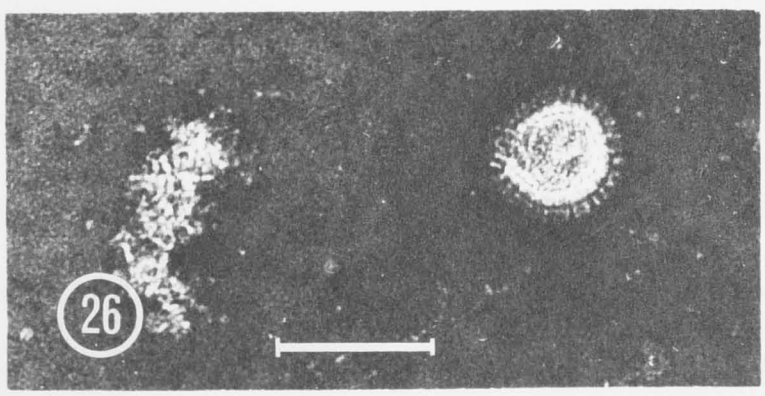
Although DOC almost abolishes infectivity of a number of influenza strains (Smith, 1939; Burnet and Lush, 1940; Sunaga et al., 1959; Webster and Laver, 1966), partially disrupted particles, such as those shown in Fig. 16 were common in DOC-treated BEL preparations, indicating that this detergent is incapable of completely disrupting this strain. This probably accounts for the failure of this detergent to produce haemagglutinating subunits of uniform size from the BEL strain (Webster and Laver, 1966). DOC also fails to produce haemagglutinating subunits of uniform size with a number of other strains (Mizutani and Mizutani, 1966; Webster and Laver, 1966) and does not permit the complete separation of neuraminidase and HA of the LEE strain by electrophoresis (Laver, 1963).

In contrast to DOC, SDS is reported to reduce BEL HA to subunits (Laver, 1964; Laver and Webster, 1966; Webster and Laver, 1966), although nearly 40% of the infectivity of BEL survived SDS treatment (Webster and Laver, 1966). Electron micrographs of BEL

treated with SDS revealed few recognizable virus particles, although many aggregates of amorphous material were present which confirmed the assumption (Laver, 1964) that on the whole BEL is disrupted by SDS (Fig. 25). Those few particles which remained intact were small and generally spherical, being 700 - 900 Å in diameter. An example at higher magnification is shown in Fig. 26.

The variable lipid composition of the MEL strain of influenza virus grown in the same host cell (Kates et al., 1962) suggests that for the assembly of the virion, the precise nature of the lipid is not important. However, stability of the virion is probably dependent upon its lipid, since infectious virions of the MEL strain grown in different host cells differ in their resistance to SDS (Burnet and Lush, 1940), while infectious virions of several strains grown in different tissues of the same host differ in susceptibility to phospholipase C (Simpson and Hauser, 1966). Filamentous virus resembles cytoplasmic membranes in several respects (Burnet and Lind, 1957) and our findings strongly suggest that pleomorphic virus too differs in some way from small spherical virus, which appears to be more resistant to SDS. The difference may lie with the lipid in the virion, since pleomorphic virus has a lipid composition which differs from that of normal virus (Blough et al. 1967). Although this may hold for the majority of the pleomorphic virus, any formed by fusion of two small SDS-resistant spherical virions might also be expected to survive treatment with SDS; an example of such a particle is shown in Fig. 27.





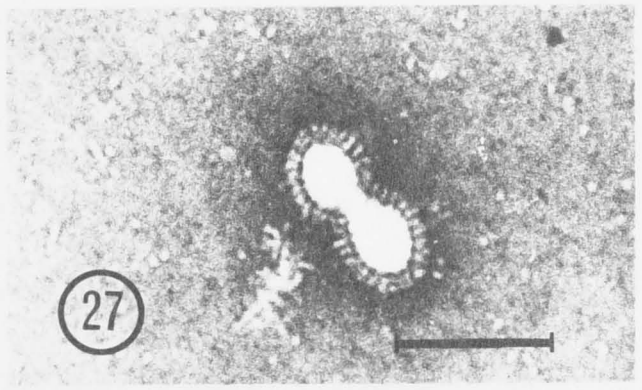


Fig. 25. Amorphous material and small intact spherical particles after treatment of BEL virus with SDS. Scale line = 2,000 Å.

Figs. 26, 27. SDS-resistant BEL virions at high magnification. Scale line = 1,000 Å.

At present it is not possible to draw conclusions on the composition or genetic behaviour of infectious influenza virus particles by studying the structure or behaviour of virus populations, since the latter contain a majority of non-infectious particles. For this reason, we do not know which of the many forms of the RNP is present in infectious virions. The ease with which 'extension spring' RNP coils are found in influenza virus preparations may be a reflection on the instability of particles containing these structures. The complete spiral of our Takátsy loop structure (which was only rarely seen) may be the form present in the more stable spherical particles and may therefore be closer to the form of the RNP present in the infectious virion, if only the spherical particles are infectious. However, it has been demonstrated that heteroploid parainfluenza virus may be infectious (Dahlberg and Simon, 1969) and this is certainly the case with influenza virus filaments (Ada et al., 1958) and probably also the case with pleomorphic influenza virus. But the smallest particle capable of harbouring a full complement of RNP makes the best use of available virion coat proteins, and would therefore be the preferred form of the infectious virion. Indeed, filamentous forms are generally replaced by spherical mutants during repeated passage of influenza virus in the allantoic cavity of chick embryos. We believe that the spherical particles shown in Fig. 25 represent the infectious particles of BEL which are known to survive SDS treatment (Webster and Laver, 1966) and the possibility of using SDS to prepare virus with a high proportion of infectious particles is currently being investigated in Melbourne.

PAPER 2.

Immediate visualisation of proteins in dodecyl sulphate-polyacrylamide gels by prestaining with a Remazol dye.

Immediate visualisation of proteins in dodecyl-sulphate polyacrylamide gels by prestaining with a Remazol dye.

Polyacrylamide gel electrophoresis has gained wide acceptance as an analytical guide to purity at different stages in the purification of proteins. Proteins have been located in the gels by staining with amido black (Davis, 1964) or by the more sensitive Coomassie blue (Maizel, 1966) followed by removal of excess dye, or by prestaining proteins with fluorescent dyes (Kierszenbaum etal., 1969). The time required to stain and destain polyacrylamide gels has been reduced by the introduction of electrophoretic destaining procedures (Peterson, 1968) or by using 0.05% Coomassie blue in 10% trichloracetic acid (Chrambach etal., 1967), but generally, several hours are required for these procedures. Recently, a rapid method for staining proteins in polyacrylamide gels has been described by Hartman and Udenfriend (1969), but this method was found to be of low sensitivity for staining gels containing SDS, as the latter produces a background fluorescence.

In this paper, a procedure is described for reacting biological material with a dye (Remazol Brilliant Blue R) during denaturation prior to analysis in dodecyl-sulphate polyacrylamide gels. The sensitivity of Remazol Brilliant Blue prestaining was better than that obtained with Coomassie Brilliant Blue alone. The method is illustrated by the rapid evaluation of purity of influenza virus fractionated in density gradients.

MATERIALS.

1. Remazol Brilliant Blue R and Remazol Red B were gifts from Australian Hoechst Ltd.
2. Coomassie Brilliant Blue R250 was a gift from Imperial Chemical Industries (Australia) Ltd.
3. Influenza virus; the filamentous recombinant $X7F_1$ of Kilbourne (Kilbourne etal., 1967), and influenza B/Vic/65 were used.
4. Buffers used were: sodium carbonate-bicarbonate (pH10, 1M), sodium carbonate-sodium dihydrogenphosphate buffer (pH 9.0, 1M), and sodium phosphate buffer (pH 8.0, 1M).

METHODS.

Virus propagation and purification.

Influenza virus was inoculated into the allantoic cavity of 10-day old chicken embryos and the allantoic fluid harvested after 46 hours. B/Vic/65 and $X7F_1$ virus were purified by two cycles of adsorption onto and elution from human erythrocytes, followed by differential, buoyant-density and rate-zonal centrifugation as will be described elsewhere (Griffith, in preparation+). High and low density virus was separated by $10\frac{1}{2}$ hour centrifugation into a preformed sucrose-tartrate buoyant density gradient. These fractions were further separated into slowly and rapidly sedimenting components using a preformed 5 - 20% sucrose gradient.

+Chapter 3

Preparation of samples for electrophoresis.

From 1 - 5 mg. (in 0.025 - 0.10 ml physiological saline) of each virus sample were mixed (where necessary) with additional saline to a final volume of 0.15 ml in screw-cap vials. SDS was added (0.15 ml of a 10%, w/v, solution) together with buffer of the appropriate pH (0.3 ml of a 1 M solution), 0.025 ml EDTA (5%, w/v in water), and 0.025 ml or 0.05 ml of a freshly prepared solution of Remazol Brilliant Blue R (100 mg/ml in 1% w/v SDS). The vials were capped and placed upright in a beaker containing a little cold water which was then brought to the boil. After five minutes, the vials were removed and 0.075 ml dithiothreitol (0.5 M) added to each sample. The samples were left to cool for 30 minutes. Solid urea was then added to just give a saturated solution and the samples left for a further 30 minutes. Iodoacetamide (0.1 ml of a 1 M solution in 1 M-sodium bicarbonate) was then added to each sample, and the mixtures left for fifteen minutes with occasional mixing at room temperature. Urea crystals were deposited by centrifugation (10 min. at 15°). Samples were applied immediately to the gel, or freed of excess stain in the following way: the sample (about 0.7 ml) was mixed with 10 ml freshly prepared acetone:methanol:acetic acid (24:12:1, by vol.). After fifteen minutes, the flocculated protein was collected by centrifugation and dissolved with warming as follows: 0.1 - 0.2 ml SDS (5% w/v, containing 0.25 mg/ml phenolphthalein) and sufficient crystals of tris base to give a purple colour were added, followed by sufficient solid urea to give a saturated solution. This

precipitation procedure leaves dye and probably viral lipid in solution. To check that all protein was precipitated, the supernates from the acid acetone-methanol precipitation of four virus samples were pooled and taken to dryness. The solids were dissolved in 2 ml water and the lipid extracted according to Bligh and Dyer (1959) with 5 ml methanol plus 5 ml chloroform followed by 2.5 ml distilled water. The chloroform and aqueous methanol phases were separated and taken to dryness. The chloroform extract was dissolved in 0.1 ml 5% SDS in 0.1 M-Tris, and dialysed for 16 hour against 0.1% SDS in 0.01 M-phosphate buffer pH 7.0 containing 6% sucrose. The aqueous methanol residue was dissolved in the minimum amount of distilled water (about 4 ml) and desalted and concentrated by dialysis against acetone-water (9:1 by vol.) containing 0.1% SDS for 16 hours. The dialysis bag contents were further concentrated by placing the bag in a stream of air at room temperature for one hour. Finally, the bag, containing about 0.05 ml of fluid, was dialysed for 2 hours against the SDS-phosphate dialysis buffer used above.

Polyacrylamide gel electrophoresis.

Electrophoresis was carried out in 10% polyacrylamide gels in 0.1 M phosphate buffer pH 7.0 containing 0.1% (w/v) SDS as described by Weber and Osborn (1969). Samples (0.01 - 0.035 ml) were layered on top of the gels and subjected to electrophoresis at 6.5 or 8 mA per tube for various times.

Procedure of fixation and staining.

Gels were removed by injection of 0.01% SDS solution between the glass tube and the gel. Gels containing prestained material were fixed and stored in acetic acid:methanol:water (1:9:10, by vol.). Other gels were stained with 0.5% (w/v) Coomassie blue in acetic acid:methanol:water (2:8:10, by vol.) overnight, and destained with agitation in acetic acid:methanol:water (1:9:10, by vol.).

Photography.

Gels were photographed before or after removal from the glass tubes, using a Wratten number 23A gelatine filter.

Results.

The activation of Remazol dyes and their reaction with protein and carbohydrate take place most readily at alkaline pH (Bohnert and Weingarten, 1967). In order to find a suitable pH, preparations of two different influenza strains were reacted with Remazol Brilliant Blue R at pH 8,9, and 10 as described in methods. As controls equivalent amounts of virus were denatured in the same manner at the different pHs, but in the absence of dye. Equal volumes of the dyed and undyed samples were applied to 5% gels and electrophoresed for 5 hours at 6.5 mA/tube. The gels were removed and those containing the prestained material were fixed in acidified aqueous methanol; the controls were stained with Coomassie blue as described in Methods. The results are shown in Fig. 1. Apart from a band of high mobility in the Remazol stained gels (which increased in amount with increasing pH) the pattern of polypeptides was

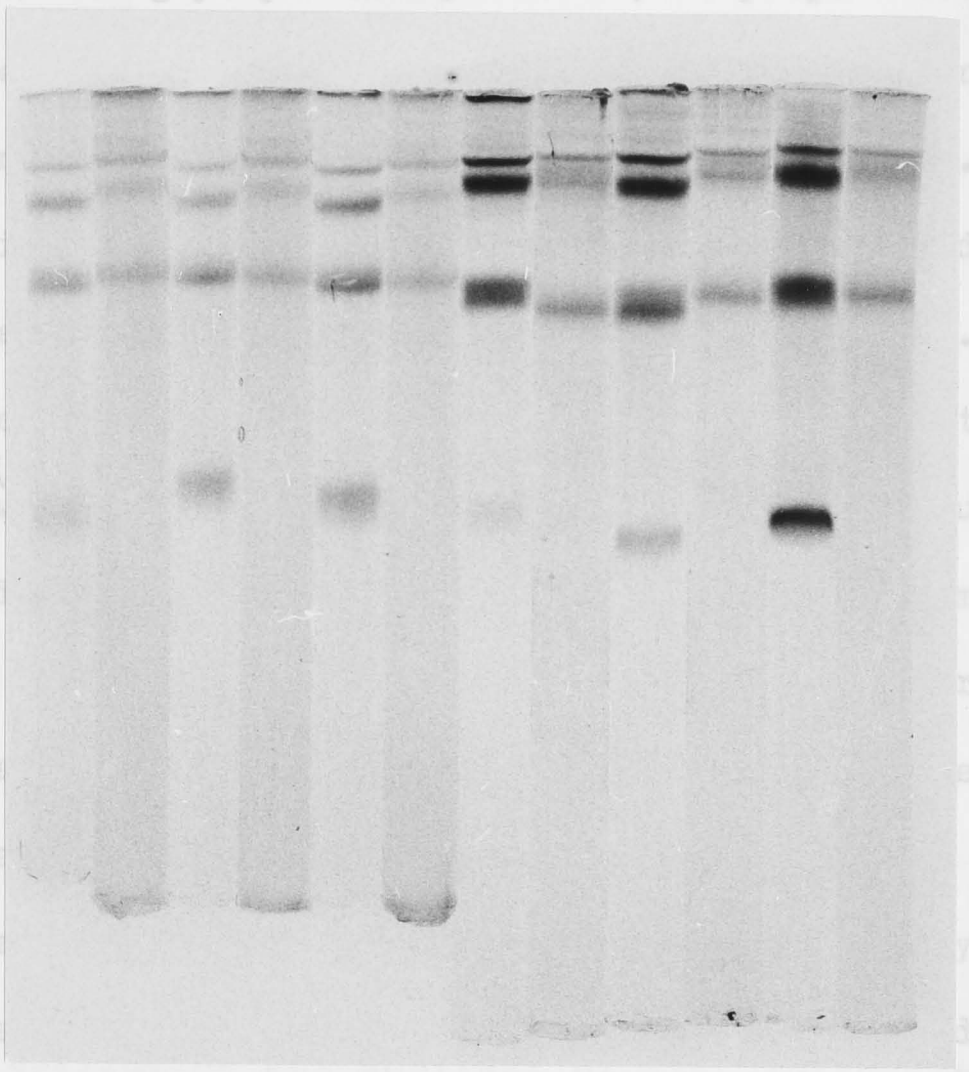


Fig. 1. Polyacrylamide gel analysis of denatured X7F$_1$ (1 - 6) and B/Vic/65 (7 - 12) influenza virus, stained with Remazol blue R (odd numbers) or Coomassie brilliant blue (even numbers). Virus was denatured at pH 8.0 (gels 1,2,7,8), pH 9.0 (gels 3,4 9,10), or pH 10.0 (gels 5,6,11,12). Virus was prestained with 2.5 mg (gels 1 - 6) or 5 mg (gels 7 - 12) Remazol blue per sample; samples were applied without removal of excess dye by precipition, and gels were stored in fixing solvent for 48 hours before photography (see text).

essentially similar in each group of six gels. The dye intensity with Remazol blue prestaining was as good as, or better than, that with Coomassie blue. It may be shown that the bands indicated by arrows also stain for glycoprotein (Griffith, in preparation[+]).

Before fixation of the prestained gels a broad band of stain was present in the gel; this diffused out on storage. In 10% polyacrylamide gels this ran between the additional band of high mobility and the most rapidly moving viral polypeptide (Fig.2a). The least mobile portion of this broad band was readily lost from the ~~gel~~ sample by ~~diffusion~~ dialysis overnight (Fig.2b) and corresponds to unreacted Remazol blue which was run in parallel (Fig.2d). The more mobile portion of this broad band was lost after a further few hours dialysis. ~~storage~~. The band of high mobility was also reduced in intensity during ~~this~~ gel fixation, but failed to completely diffuse from the gel even on prolonged storage. It was thought that this rapidly moving band might represent stained viral lipid. It was considered that precipitation of the protein with acidified methanolic acetone would remove both the lipid and the excess stain, and this appeared to be the case (Fig.2c).

After precipitation of the stained protein, the acidified methanolic acetone supernate was separated into 'lipid' and 'non-lipid' fractions as described in Methods. These fractions were electrophoresed in parallel with stain which had been heated to 100° for 15 minutes at pH 9.0. Fig. 2e shows that the 'non-lipid' fraction contained no high molecular weight viral proteins, but did

[+]Paper 5

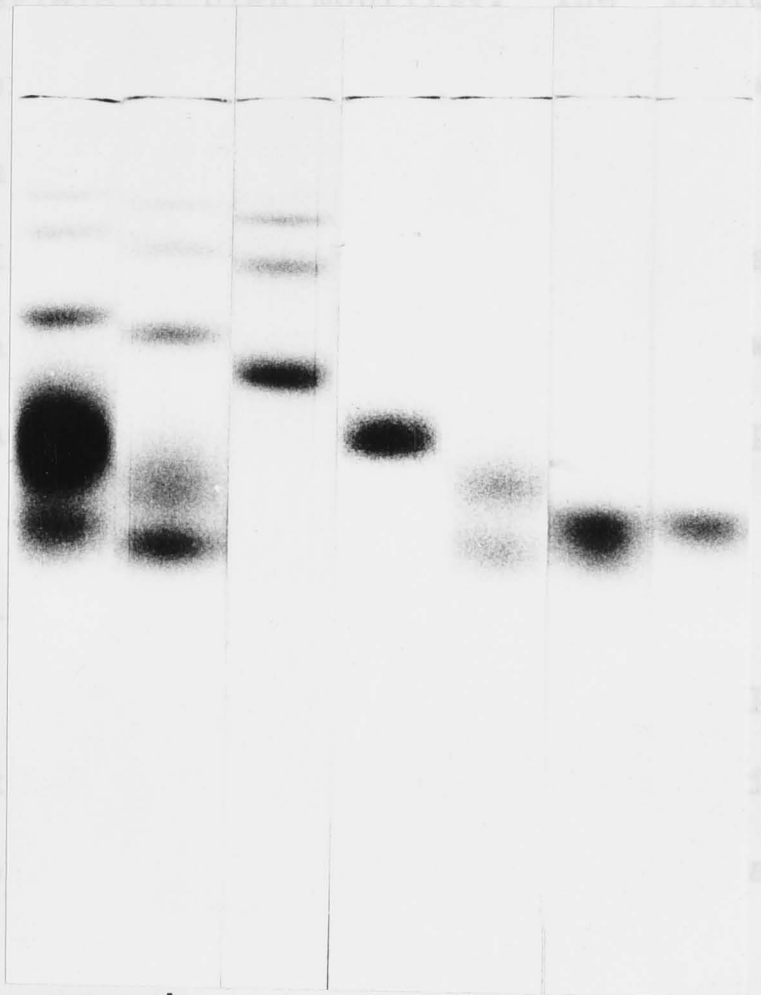


Fig. 2. Polyacrylamide gel analysis of X7F$_1$ influenza virus. (a - c), virus sample reacted with excess Remazol stain (a) which partially diffused from the sample during overnight dialysis (b), or was separated by precipitation of the protein before analysis (c); (d), unactivated Remazol dye; (e,f), 'non-lipid'(e) and 'lipid' (f) fractions of the solvent used for protein precipitation; (g) activated Remazol dye.

contain stained materials of high mobility. The 'lipid' fraction contained the band of high mobility (Fig.2f) but this was also present in dye which had been activated for 15 min. at 100° at pH 10.0 (Fig.2g). It is possible therefore that the band of high mobility is merely a reaction product of the dye. It was observed that it was more readily formed at higher pH at the expense of the less mobile rapidly diffusing stain (See Fig. 1).

These preliminary experiments indicated that the viral protein was readily stained at pH 9.0 and could be completely recovered free of excess stain (and presumably viral lipid and Remazol blue reaction products) by precipitation with acidified methanolic acetone.

The ability of the technique to allow rapid assessment of viral purity is illustrated in Fig.3. Partially purified virus was separated into high and low density fractions, which were further divided by rate-zonal centrifugation into 'slow' and 'fast' sedimenting fractions, as will be described elsewhere (Griffith, in preparation). These four fractions 'high density slow', 'high density fast', 'low density slow', 'low density fast' were treated with Remazol blue at pH 9.0, freed of excess dye by precipitation, redissolved in SDS-urea buffer and applied to 10% polyacrylamide gels as described in Methods. After electrophoresis for 4 hours at 8 mA/tube it was evident that the 'low density' fractions contained one polypeptide (arrowed, Fig. 3 a,b) not present in the 'high density' fractions (Fig.3 c,d). This contaminating polypeptide

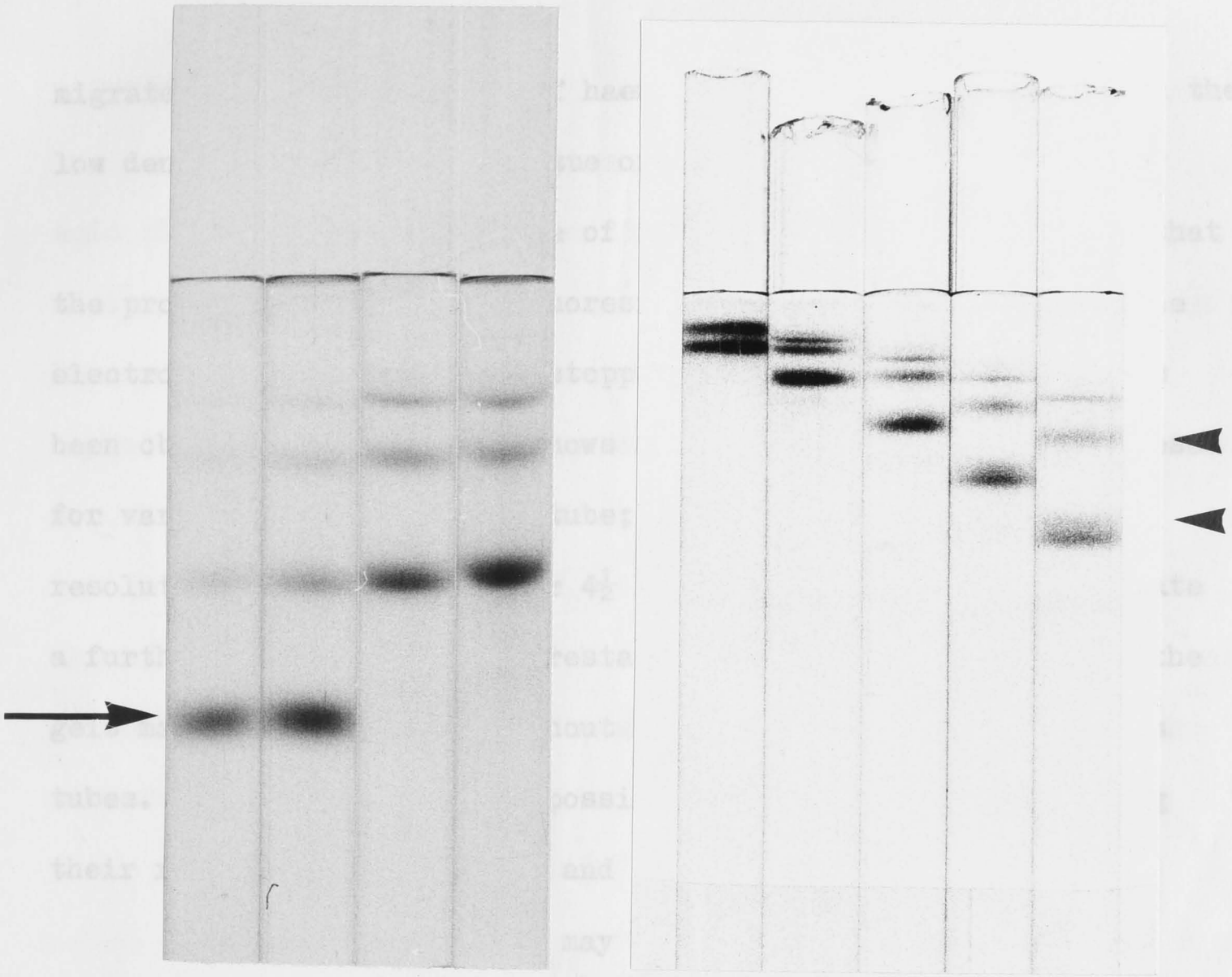


Fig. 3. Analysis in polyacrylamide gels of different virus ($X7F_1$) containing fractions isolated during virus purification. Low density (a,b) and high density (c,d) virus separated into fast (a,c) and slow (b,d) sedimenting fractions in density gradients; the arrow indicates contaminating haemoglobin polypeptides. The high density, slowly sedimenting fraction of $X7F_1$ virus electrophoresed for (e) 0.75, (f) 1.5, (g), 2.5 (h) 3.5, (i) 4.5 hours at 6.5 mA per tube; bands staining for glycoprotein are indicated by arrow heads.

migrated with the mobility of haemoglobin, which was visible in the low density fractions by virtue of its red colour.

A further advantage of the prestaining technique is that the progress of the electrophoresis may be followed by eye. The electrophoresis may then be stopped when maximum resolution has been obtained. Fig. 3 e-i shows a sample of virus electrophoresed for various times at 6.5 mA/tube; it is evident that maximum resolution was achieved after $4\frac{1}{2}$ hours. Figs. 2 and 3 illustrate a further advantage of the prestaining technique, namely that the gels may be photographed without being removed from their glass tubes. This eliminates the possible destruction of gels during their removal from the tubes and subsequent staining.

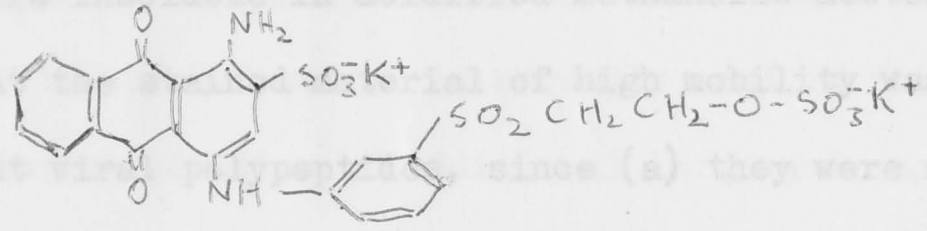
Other Remazol dyes may also be used for the staining procedure described in this paper. It is sometimes an advantage to run standard proteins as reference markers in acrylamide gels, and glucagon stained with Remazol Red B has been used for that purpose in this laboratory.

DISCUSSION.

The binding of Coomassie blue by proteins at slightly acid pH is by an electrostatic attraction which is stabilised by additional Van de Waal's forces (Fazekas de St. Groth *etal*. 1963). In contrast, the binding of the activated Remazol dyes is by covalent linkage (Bohnert and Weingarten, 1967). Remazol dyes are sulfatoethyl sulfones having the general structure

$$R - SO_2 - CH_2 - CH_2 - O - SO_3^-$$

where, in the case of Remazol Brilliant Blue, R is an anthraquinone sulfonic acid. This dye was shown by Panchartek *etal*. (1962) to have the structure



At alkaline pH it is converted to its vinyl sulfone derivative

$$R\text{-}SO_2\text{-}CH_2\text{-}CH_2\text{-}O\text{-}SO_3^- + OH^- \longrightarrow R\text{-}SO_2\text{-}CH = CH_2 + SO_4^{=} + H_2O$$

which reacts rapidly under the conditions of digestion with any primary and secondary amines, alcoholic OH groups and SH groups (Bohnert and Weingarten, 1967). Thus, in addition to staining protein, Remazol dyes will react with carbohydrates, nucleic acid and complex lipids. In side reactions the activated dye reacts with water giving the hydroxyethylsulphone (Bohnert and Weingarten, 1967). It is also possible that the activated dye molecule reacts with itself, with the NH_2 group of the chromophore, or in a manner analogous to acrylamide, yielding dye polymers of variable lengths.

These will have the high negative charge of the chromophore. Since the dye (both before and after activation) is soluble in methanolic chloroform, and more readily soluble in 1% SDS solution than distilled water (unpublished observations), it is probably somewhat hydrophobic in character. Since the Remazol dye has a negatively charged chromophore, after reaction with the dye, the positively charged residues in the protein will have been converted to negative charges. The resulting high negative charge of the reacted protein will probably render it insoluble at acid pH. Indeed, all the Remazol stained high molecular weight viral polypeptides were insoluble in acidified methanolic acetone. It is unlikely that the stained material of high mobility was low molecular weight viral polypeptides, since (a) they were not present in the Coomassie blue stained controls (b) this material almost all diffused from the gels within 48 hours of fixation (c) Remazol stained glucagon (molecular weight 3,500) was found to be insoluble in the acidified methanolic acetone and did not diffuse from the gels on storage . This stained material of high mobility, which was soluble in the acidified methanolic acetone, is thus likely to be dye reaction products, and possibly stained viral lipids. Its high mobility indicates that it was of low molecular weight and had a high negative charge, perhaps both intrinsic and through complexing with the SDS. However, its failure to completely diffuse from the gels indicates that it was not all small molecules. Since the protein is freed of buffer salts as well as excess dye

(and its postulated reaction products) by the precipitation step, the latter is routinely used in this laboratory. Where doubt remains about the efficacy of the precipitation step, samples may be electrophoresed without precipitation, and freed of all but a small fraction of excess dye by the gel fixation procedure.

The Remazol dye was found to react with protein under the three pH conditions tested. Since hydroxyl ions are consumed during activation of the dye, a high concentration of buffer is necessary. Even so, the pH may drop during the reaction; for example, with the pH 8.0 buffer, the pH fell to 7.6. Since the proteins become negatively charged on staining, and since the reaction is favoured by alkaline pH, the pH 9.0 buffer is normally used in this laboratory. The introduction of additional large and negatively charged chromophore groups probably assists denaturation by unfolding the polypeptides by charge repulsion effects. Staining of material at pH 10 is not advisable, since glycoproteins may be fragmented at this pH at elevated temperatures (Neuberger and Marshall, 1966).

It is perhaps surprising that the mobilities of the polypeptides stained with Remazol are not altered to any great extent when compared with those of the untreated controls stained with Coomassie blue (Fig.1). Presumably the expected decrease in mobility resulting from an increase in molecular weight (corresponding to the amount of dye bound) is offset by an increase in mobility due to the increased negative charge on the stained

polypeptides. These factors may not necessarily cancel each other out, as the smaller glycopeptide appears to migrate more slowly after reaction with the Remazol dye. Influenza contains, in addition to viral protein and glycoprotein rather resistant to denaturation, lipid and RNA. The glycoprotein is present as two polypeptides in the gels of the pure virus as indicated in Fig. 3. The superior staining of the glycopolypeptide bands with Remazol blue is evident in Fig.1. The faint blue band of material which barely penetrates the top of the gel may possibly represent that portion of viral RNA which has not been hydrolysed.

The prestaining procedure described has several advantages over present methods of visualising proteins in polyacrylamide gels. In addition to being a stain of greater sensitivity than Coomassie blue, Remazol blue appears to be a superior stain for assessing the purity of material such as influenza virus which is of complex chemical structure. Additional advantages are gained by use of the prestaining procedure. Where protein is freed of excess stain by precipitation with acidified methanolic acetone, the colour intensity of the redissolved protein precipitate is a measure of the amount of material present, and the optimal amount may therefore be applied to the top of acrylamide gels without prior, time-consuming, protein estimations. The need for tracing dyes, such as bromophenol blue, is also eliminated. The progress of the electrophoresis may be followed, giving results within an hour of sample application to the gel, and electrophoresis may be stopped when maximum resolution

has been achieved. If the migrating bands are observed to 'sag', due to over-heating of the gel in its centre, the applied voltage may be reduced. The gels may be photographed immediately, and possible breakage accompanying removal of the gel from the tube is eliminated. Where necessary, gels may be removed from the tubes and fixed, and stored in appropriate solutions without loss of band intensity due to elution of the stain from the protein. However, the greatest single advantage of the method described is the elimination of time-consuming staining and destaining procedures, and the need for special apparatus to do this.

PAPER 3.

The preparation of highly infectious influenza virus.

Ever since it was demonstrated that the allantoic cavity of chicken embryos may be used to propagate influenza virus in quantity (Smith, 1935; Burnet, 1935), the purification of the virus from infected allantoic fluid has attracted considerable attention. A variety of initial concentration procedures have been developed over the years, and these have been compared by Veeraraghavan and Streevalsan (1961) and Reimer et al., (1966). Most of these procedures are suitable for concentration of a variety of strains, but not all lend themselves to the preparation of highly infectious virus. Indeed, none yield virus with a particle to infectivity ratio lower than 10:1, principally because much non-infectious virus is produced by infected cells (Isaacs, 1957), but also because influenza virus is readily inactivated by heat (Horsfall, 1955), light and oxygen (Appleyard, 1967), and heavy metal ions (Allison et al., 1962).

Webster and Laver (1966) demonstrated that much of the infectivity of the BEL strain of influenza Ao remained after treating virus with the ionic detergent sodium dodecyl sulphate (SDS). Examination with the electron microscope of a BEL virus preparation treated with SDS indicated that the majority of the pleomorphic particles were disrupted and that the intact particles remaining were mostly small and spherical (Griffith and Wrigley, in preparation); it was thought that the surviving particles might be the infectious virions of the BEL strain.

This paper describes the purification of the BEL strain by

adsorption-elution on aluminium phosphate, followed by differential, buoyant density and rate zonal centrifugation. The purified virus was treated with SDS, which appeared to selectively destroy non-infectious virus, leaving virus with a low particle to infectivity ratio.

MATERIALS AND METHODS.

Chemicals.

Sucrose, $NaH_2PO_4.2H_2O$, Na_2HPO_4 and potassium sodium tartrate were A.R. grade, and $AlCl_3.6H_2O$ was L.R. grade; all were obtained from British Drug Houses, Poole, Dorset. $Na_3PO_4.12H_2O$ was obtained from May and Baker, Dagenham. Glycerol (redistilled G.R.) was obtained from Merck, and cysteine (puriss) from Fluka. Sodium citrate (A.R.) was obtained from Standard Laboratories Pty. Ltd., Melbourne. Sodium dodecyl sulphate was obtained from Matheson, Coleman and Bell and was used as a 10% (w/v) aqueous solution. Tween 80 was obtained from Atlas Powder Co., Wilmington, Delaware; it was deionised by storage over mixed-bed resin and used as a 10% (v/v) aqueous solution. Polystyrene latex (2500 Å mean diameter) was a gift from the Monsanto Chemical Co.

Virus.

The virus used was BEL, a type Ao strain of influenza virus (Burnet et al., 1942). Seed virus was diluted 10^{-5} in Ca-Mg saline (Fazekas de St. Groth et al., 1958) and about $10^4 ID_{50}$ of the virus were inoculated into the allantoic cavity of 10 - 11 day old chick embryos. After 36 hours incubation at 33°, the eggs were chilled at 4° for 9 hours. Allantoic fluid was harvested into bottles containing 300 mg cysteine per 100 eggs used.

Preparation of aluminium phosphate.

Batch-adsorption-elution on aluminium phosphate gel has been successfully applied to a number of strains of influenza virus

(Miller and Schlesinger, 1955; Frommhagen and Knight, 1959; Veeraraghavan and Streevalsan, 1961). In preliminary experiments it was noted that the aluminium phosphate gel prepared by the method of Miller and Schlesinger (1955) was converted to a very fine suspension when mixed with a large volume of distilled water; the gel was again formed on the addition of sodium phosphate buffer (pH 8.0) to a final molarity of about 0.09. The batch adsorption-elution procedure was thus modified to make use of the greater adsorbing capacity of finely divided aluminium phosphate.

Sodium phosphate (80 g) was dissolved in 4 litres of distilled water. To this was added $AlCl_3$ (53g) dissolved in 4 litres of distilled water. A few crystals of phenol red were added and the pH of the mixture adjusted to a phenol red end-point by the gradual addition of a saturated solution of sodium phosphate. The $AlPO_4$ was collected by centrifugation (International PR2, head 276a, 2,300 rev/min., 15 min.) and suspended in 3,250 ml distilled water; the $AlPO_4$ was flocculated by the addition of 300 ml 1 M-sodium phosphate buffer, pH 6.0, and collected by centrifugation. The gel was washed a second time in the same way, but then resuspended in distilled water to a final volume of 2 litres. The stock suspension was stored at 4^{o} in the presence of sodium azide (0.05% w/v).

Concentration of virus.

To each litre of allantoic fluid were added: 250 mg cysteine, 25 ml 1 M-sodium phosphate buffer (pH 6.0), and 500 ml

distilled water followed by 100 ml of the stock aluminium phosphate suspension; all except the phosphate buffer were chilled to less than 4° before mixing. After 30 min. at 0°, the $AlPO_4$ was collected by centrifugation, washed once with a volume equal to that of the original allantoic fluid of ice-cold 0.1 M-NaCl (containing 0.01 M-phosphate buffer, pH 6.0). For each litre of allantoic fluid, the virus was eluted at 0° from the gel with 40 ml 0.30 M-sodium phosphate buffer (pH 8.0), 120 ml of a mixture of equal volumes of this buffer and 0.25 M-sodium citrate, and 60 ml 0.25 M-sodium citrate. Elution with sodium citrate converts the gel to a fine suspension of aluminium phosphate; this was assisted by a single cycle of homogenisation with a teflon-in-glass homogeniser.

Each eluate was centrifuged in a Christ centrifuge Zeta 17 (8710 head, 8000 rev/min., 15 min.). The pooled eluates were concentrated by centrifugation in a Spinco 21 L rotor (19,000 rev/min., 55 min.) onto a glycerol-sucrose-tartrate (G.S.T.) cushion (0.5 ml of the light component of the buoyant density gradient described below). Pelleted virus was resuspended by gently pipetting it in a little Ca-Mg saline (Fazekas de St. Groth et al., 1958) containing cysteine (1 mg/ml); deionised Tween 80 was added to a final concentration of 2% (v/v).

Density gradient centrifugation.

Buoyant density gradients were prepared from a glycerol-sucrose-tartrate mixture. A stock solution was prepared consisting

of a mixture of 22.1 g sucrose, 22.1 g Na-K tartrate and 35 ml glycerol, 0.08 g sodium azide, 25 mg cysteine plus distilled water to 100 ml. 30.9 ml portions of this GST stock solution diluted with 5.1 ml and 23.1 ml distilled water comprised the 'heavy' and 'light' components of the density gradient. 3 ml of the stock solution were placed in the bottom of a 3 by 1 inch Spinco tube, and on top was layered a linear gradient prepared from 11 ml heavy and 12 ml light components with an appropriate gradient forming device. After chilling the gradients, 3 - 5 ml virus concentrate was layered on top, and the tubes centrifuged for 9.5 hours at 23,000 rev/min., (Spinco SW 25.1 swing-out head). Thirty 1 ml fractions were collected through a hole punctured in the bottom of the tube to one side of the $AlPO_4$ pellet.

The fractions containing the virus were pooled, diluted with an equal volume of ice-cold, distilled water, and pelleted onto the tube surface (Spinco 30 rotor, 25,000 rev/min., 40 min) or centrifuged in the SW 25.1 rotor (23,000 rev/min., 90 min.) onto a G.S.T. cushion (4.0 ml of G.S.T. stock). The virus at the interface was collected through a hole in the bottom of the tube and dialysed at 0° for 5 hours (with two changes) against 200 vol. Ca-Mg saline containing 0.25 mg/ml cysteine.

Rate zonal centrifugation.

Samples of dialysed virus were treated at 15° with SDS or at 0° with Tween 80, and centrifuged into a 10 - 30% (v/v) glycerol gradient in 0.15 M-ammonium acetate containing 0.25 mg/ml cysteine

for 80 min., at 11,000 rev/min., (Spinco SW 25.1 head). The gradients were prepared and run at 15° without pre-cooling the rotor; this procedure separates the virus from the detergents. If operations are carried out at low temperatures, SDS comes out of solution and carries the virus with it.

Titration of virus.

(a) Particle counts.

1 ml of a 50% suspension of latex particles was suspended in 20 ml distilled water and centrifuged in a bench centrifuge (ECCO type EII/10 for 15 min at maximum speed) to remove gross aggregates. The clarified suspension was diluted to 200 ml with distilled water and filtered through a Millipore filter (pore size, 450 mμ) to remove fine aggregates. Aliquots of the filtered suspension were taken to dryness and weighed. The latex particle number per ml was estimated from the known density and dimensions of the particles. This stock was used to estimate virus particle numbers by the "loop drop" method of Watson et al., (1963). Virus samples for particle counting were dialysed against 25% glycerol in 0.15 M-ammonium acetate containing 0.25 mg/ml cysteine. Appropriate dilutions of virus were mixed with an aliquot of the latex suspension, applied to a carbon-coated grid, and dried under vacuo. The grids were then stained with a little 2% phosphotungstic acid (pH 7.0) and air dried. Samples were examined with a Siemens Elmiskop II operating at 80 KV.

(b) Haemagglutinin titrations were carried out as described by Webster (1965).

(c) Infectivity was assayed in surviving pieces of allantois-on-shell, according to the method of Fazekas de St. Groth and White (1958).

Estimation of protein.

Protein was estimated by the method of Lowry et al., (1951) using bovine serum albumin as a standard.

Treatment of virus with SDS

Purified suspension (freed of Tween 80 by dialysis against Ca-Mg saline) was brought to 5% with respect to bovine serum albumin. Sufficient 10% SDS solution was added to this virus suspension to just clear the opalescence. The virus was immediately layered on a 23 ml linear sucrose gradient (5-20% w/v in Ca-Mg saline) and separated from low molecular weight material and excess SDS by centrifugation at 15° onto a 3.5 ml 60% sucrose cushion (60 min., 23,000 rev./min., Spinco SW 25.1 head). The virus band at the bottom of the gradient was recovered, applied to a GST gradient, and centrifuged for 8 hours at 23,000 rev./min. to separate virus from partially degraded virion fragments.

Acrylamide gel electrophoresis

About 1 mg of each virus sample was freed of glycerol and ammonium salts by dialysis against Ca-Mg Saline. The virus was denatured and prestained with Remazol brilliant blue R as described elsewhere (Griffith, in preparation)+. Stained material was analysed in 10% polyacrylamide gels using the procedure of Weber and Osborn (1969).

+ Paper 2

TABLE 1.

Stage in purification[a]	HA units (total)	Recovery %	HA/mg protein	ID_{50} (total)	ID_{50}/HA	Particles (total)	Particles per ID_{50}
Infected allantoic fluid	$10^{6.90}$	100	$10^{3.1}$	$10^{13.05}$	$10^{6.15}$	NT	-
Combined $AlPO_4$ eluates	$10^{6.865}$	91.2	$10^{4.3}$	NT[b]	-	NT	-
Eluate concentrate	$10^{6.83}$	85	$10^{5.21}$	$10^{12.87}$	$10^{6.04}$	NT	-
G.S.T. gradient:							
fractions 1 - 10	$10^{5.60}$	5	$10^{5.30}$	NT	-	NT	-
11 - 22	$10^{6.62}$	73	$10^{5.31}$	$10^{12.58}$	$10^{5.96}$	NT	-
23 - 25	$10^{5.38}$	3	$10^{5.28}$	NT	-	NT	-
26 - 29	$10^{5.05}$	1.5	$10^{2.60}$	NT	-	NT	-
Tween 80-treated virus:							
10 - 30 % glycerol gradient	-	-	$10^{5.29}$	-	$10^{6.00}$	NT	-
SDS-treated virus: freed of SDS and virion fragments.	$10^{5.74}$	6.9	NT	$10^{12.09}$	$10^{6.35}$	$10^{12.64}$	3.6

[a] For details, see text.

[b] NT = not tested

Results.

The activity and recovery at various stages in the purification of virus by adsorption-elution on aluminium phosphate, followed by differential, buoyant density and rate-zonal centrifugation are given in Table 1. Recovery of haemagglutinin (HA) on elution from the aluminium phosphate was virtually quantitative and virus was purified 15 fold with respect to protein. Concentration of the eluate by centrifugation was accompanied by small losses in both HA and infectivity, but the amount of contaminating material was again substantially reduced by this step. The concentrated virus was treated with Tween 80 and centrifuged to equilibrium in a GST gradient as described under Methods. The appearance of the gradient after 9.5 hours centrifugation is shown in Fig. 1 and the distribution of HA in Fig. 2. A large amount of non-haemagglutinating material remained at the top of the gradient. The material in fractions 1-10 and 26-29 was concentrated by 'pelleting', and that in fractions 11-22 and 23-25 by centrifugation onto a GST cushion as described under Methods. It is apparent from Fig. 2 that the bulk of the virus was present as a single broad band.

The concentrated material containing the bulk of the virus was divided into four unequal portions. About $10^{4.55}$ HA units were treated with Tween 80 (final detergent concentration 1% v/v) and $10^{4.85}$ HA units with SDS (final concentration 1% w/v). These were layered, together with a portion of untreated virus, on separate 10-30% glycerol gradients. These were centrifuged as described under Methods

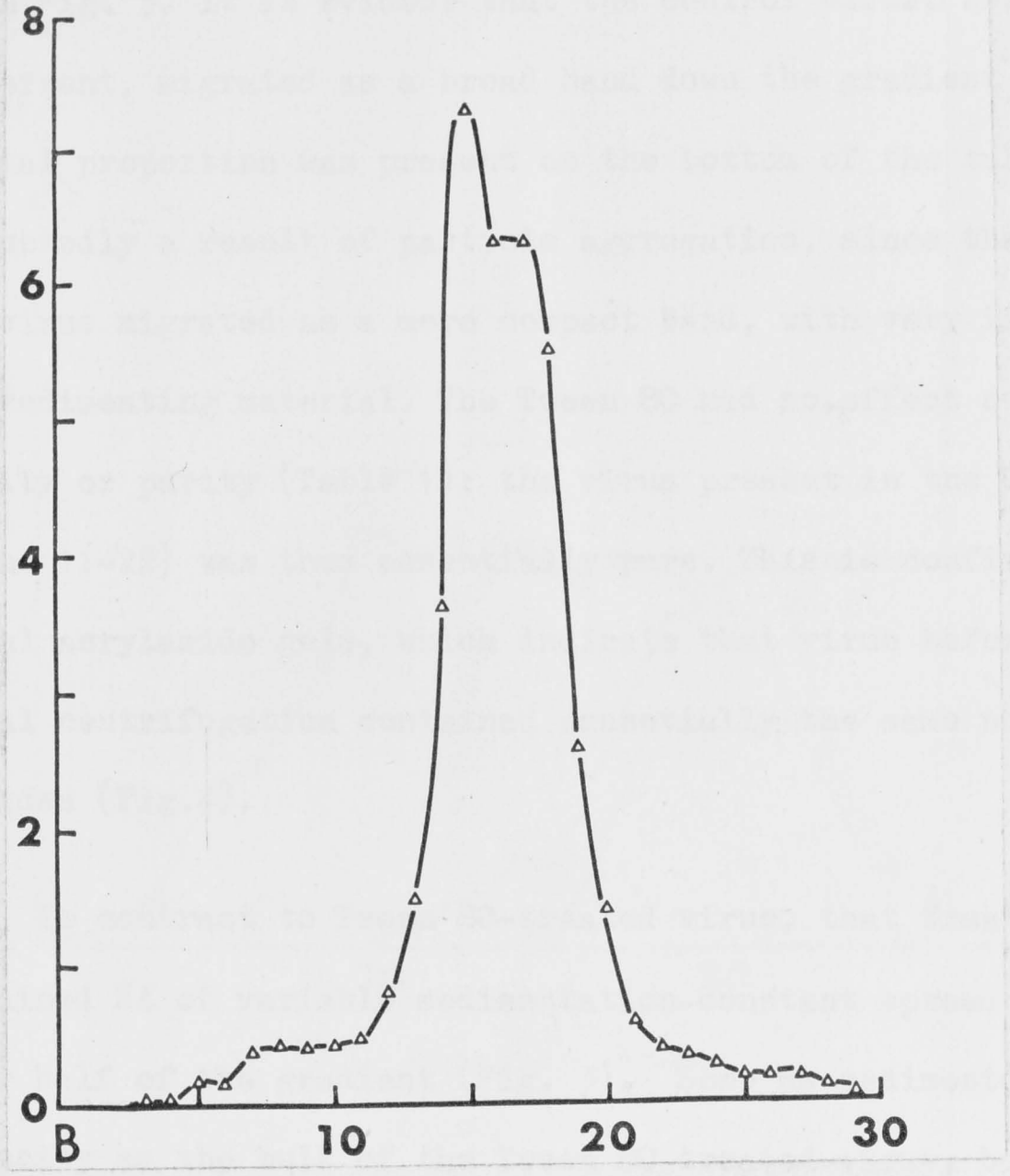


Fig. 2. Distribution of HA activity in the buoyant density illustrated in Fig. 1. Thirty 1-ml fractions were collected through a hole in the bottom (B) of the tube, and titrated for HA

and 30 one-ml fractions collected and assayed for HA activity. The distributions of the HA in the rate-zonal gradients are illustrated in Fig. 3. It is evident that the control virus, not treated with detergent, migrated as a broad band down the gradient and a substantial proportion was present on the bottom of the tube. This was undoubtedly a result of particle aggregation, since the Tween 80-treated virus migrated as a more compact band, with very little rapidly sedimenting material. The Tween 80 had no effect on virus HA, infectivity or purity (Table 1); the virus present in the GST gradient (fractions 11-22) was thus essentially pure. This is confirmed by analytical acrylamide gels, which indicate that virus before and after rate-zonal centrifugation contained essentially the same number of polypeptides (Fig.4).

In contrast to Tween 80-treated virus, that treated with SDS contained HA of variable sedimentation-constant spread throughout the upper half of the gradient (Fig. 3). Some HA sedimented with the same velocity as the bulk of the Tween 80 treated-virus, but most remained at the top of the gradient. This suggested that some virus remained intact and that some was partially degraded, but that the bulk was reduced to low molecular weight haemagglutinating subunits. It was apparent that the rate-zonal gradient failed to cleanly separate any remaining intact virus from partially disrupted virions. The rapidly sedimenting material was separated from the bulk of the virion fragments by centrifugation onto a sucrose cushion, and from large virion fragments by buoyant density centrifugation as described

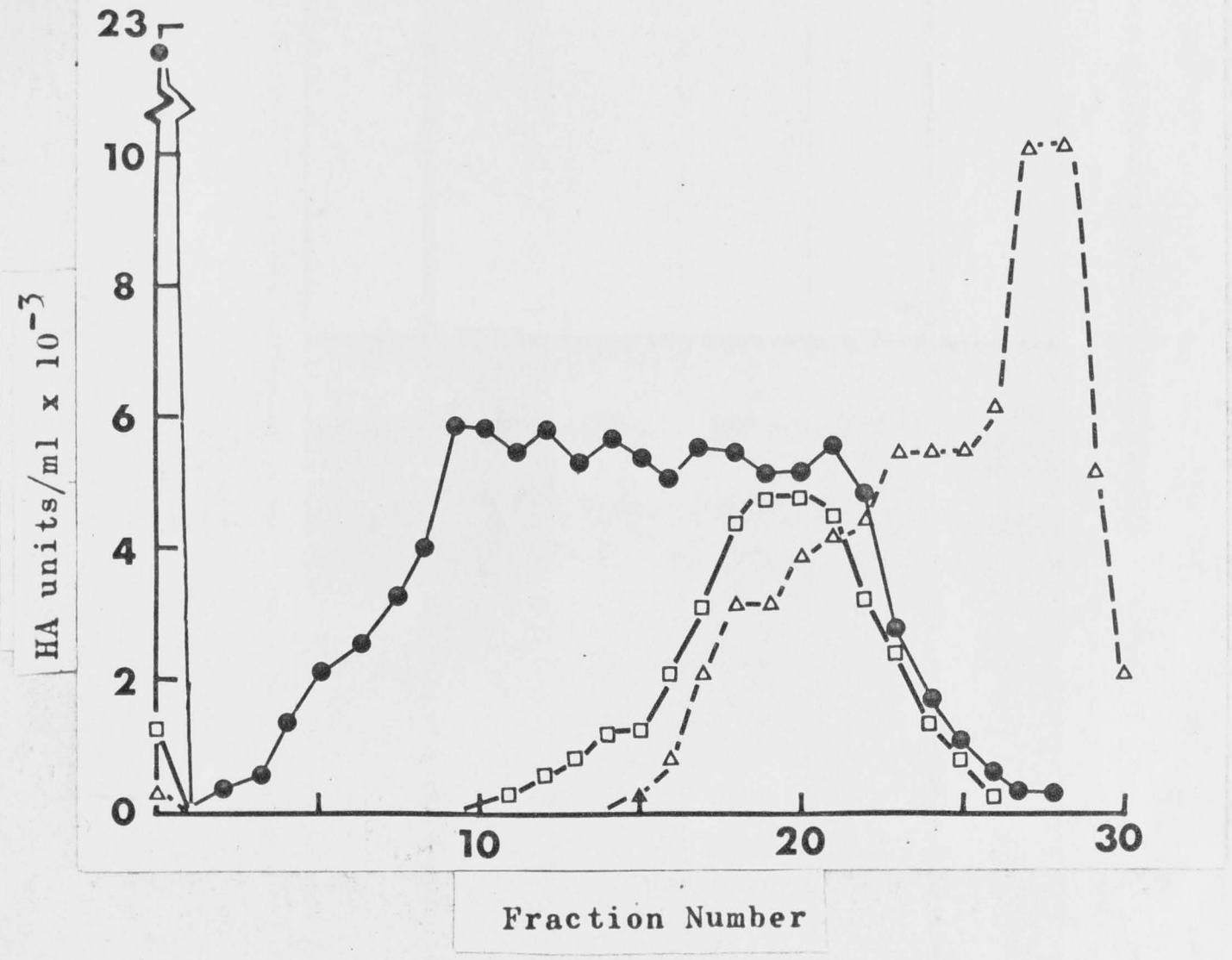


Fig. 3. Rate-zonal profile of virus in glycerol gradients. Untreated virus (filled circles), virus treated with Tween 80 (open squares), and virus treated with SDS (open triangles) were centrifuged for 80 min. at 11,000 rev./min. into 25 ml linear 10 - 30% glycerol gradients in a Spinco SW25.1 head at 15°. Thirty 1-ml fractions were collected through a hole in the tube to one side of the pellet; the latter was resuspended in the bottom fraction (B).

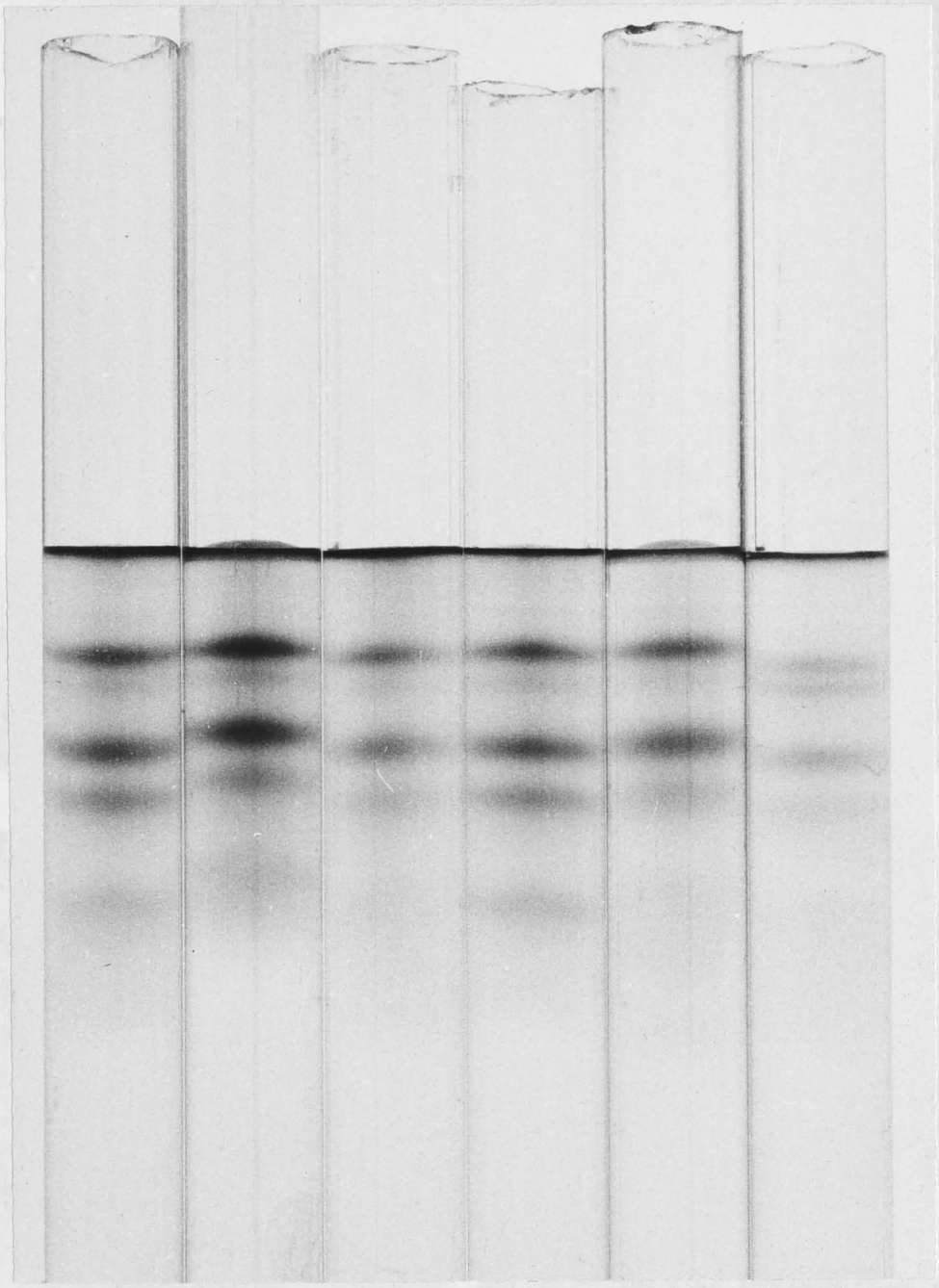


Fig. 4. Analysis in polyacrylamide gels of samples of BEL virus at different stages of purification (see Table 1). (a) eluate concentrate; (b - e), buoyant density gradient: fractions 1 - 10 (b), 11 - 22 (c), 23 - 25 (d), 26 - 29; (f), virus banded in a 10 - 30% glycerol gradient. Samples denatured with SDS were stained with Remazol brilliant blue R at pH 9.0 and 100°, followed by reduction with dithiothreitol. Samples were electrophoresed into polyacrylamide gels for 2 hours at 8 mA/gel.

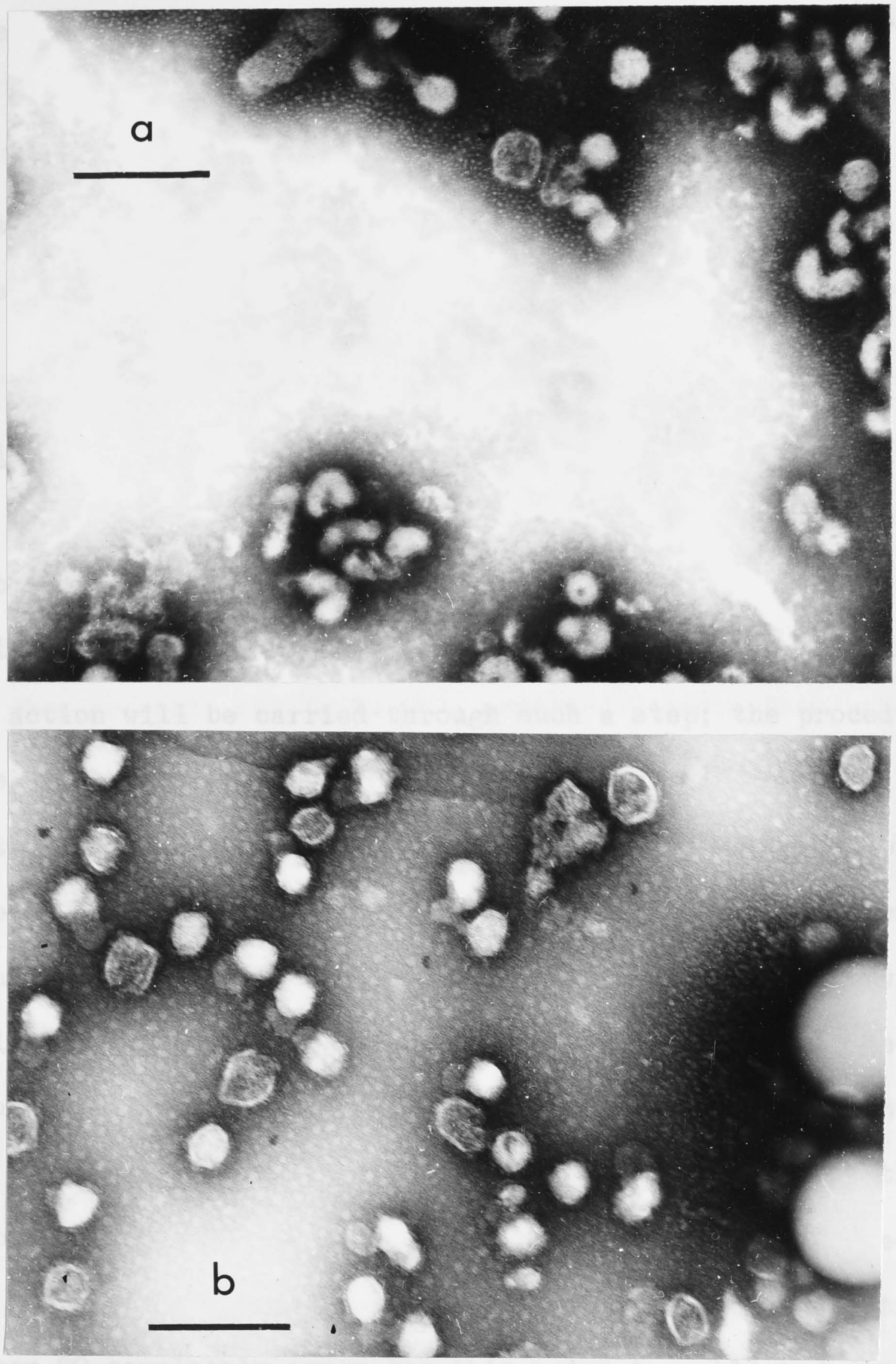


Fig. 5. Appearance of BEL virus before (a) and after (b) treatment with SDS and separation from virion fragments. Virus was negatively stained with 2% neutralised phosphotungstic acid. Bar = 2,500 Å.

under Methods. The fractions 11-22 of the latter gradient were pooled, dialysed and virus activity estimated. It is evident from Table 1 that the recovered virus had a low particle to infectivity ratio, suggesting that a proportion of infectious virions were more resistant to SDS disruption than the bulk of the non-infectious virus.

Discussion.

Purification of influenza virus by adsorption-elution with erythrocytes has frequently been used in the past, since only materials able to adsorb to red cells and to elute therefrom under enzymic action will be carried through such a step; the procedure is thus one highly specific for viral proteins. However for the preparation of infectious virus it is unsuitable, since elution is performed at room temperature, or at 37°. Furthermore some haemolysis accompanies such elution, and the virus becomes contaminated with red cell debris. This debris is only partially removed by rate-zonal centrifugation; complete removal requires an additional buoyant density centrifugation step (Griffith, in preparation)[+]. In either step substantial viral activity remains associated with the red cell debris, and neither "ageing" (Laver, 1969) nor sonication or treatment with Tween 80 completely separates this union (unpublished observations); the reason for this firm aggregation is not clear, but may be a consequence of the ability of influenza virus to fuse with cell membranes (Hoyle et al 1962; Hoyle, 1962; Morgan and Rose, 1968). For the BEL strain, adsorption-elution with red cells is unsatisfactory

[+]Chapter 3

since this strain actively lyses many of the cells. This property seems to be associated with the virion, since virus purified by the procedure described in this paper lyses erythrocytes almost as much as the infected allantoic fluid (unpublished observation).

Although adsorption-elution with aluminium phosphate does not separate virus from non-haemagglutinating non-infectious membraneous material present in infected allantoic fluid (Frommhagen and Knight, 1959), they are readily separated by buoyant density centrifugation, since these membraneous contaminents of the virus are of much lower density and remain at the top of the gradient. Red cell debris, when present, sediments to a position between this material and the main band of virus.

Detergents have been used to free virus from cell debris, Igepon T73 being used with Barley stripe mosaic virus (Brakke, 1959) and Tween 80 with rubella virus (Magnusson and Skaare, 1967); Tween 80 has also been used to achieve full recovery of influenza virus adsorbed to columns (Bengtsson and Philipson, 1964). It is evident in Fig. 3 that influenza virus recovered from a GST gradient concentrated on a GST cushion is substantially aggregated, since HA is spread through much of the rate-zonal gradient and is present in the pelleted material. These aggregates are most probably clumped virus particles, since virtually no material was pelleted after treatment of the virus with Tween 80. The absence of haemagglutinin at the top of the rate-zonal gradient indicates that this detergent, unlike SDS, does not reduce even a portion of the virus to sub-units.

The infectivity of two Asian strains (A_2/SA/64 and A_2/NT/68) was, like BEL, unaffected by exposure to excess Tween 80 at 0° for up to 3 days (unpublished observations).

Despite the care taken to preserve the infectivity of the virus during purification (by using, wherever possible, cold glycerol solutions containing cysteine as an anti-oxidant), some losses nevertheless occurred. The reason for this is not clear, but one possibility is that particle numbers have been reduced by physical fusion of virions, as suggested by Griffith and Wrigley (in preparation[+]), or by fusion of virions with cell membrane contaminants as suggested above. We have seen that SDS converts much of the virus to subunits, and that those virions resistant to the detergent are mostly small and spherical. The ability of SDS to selectively disrupt the majority of the pleomorphic virions without an equivalent decrease in the number of infectious particles, suggests that the pleomorphic virus constitutes the bulk of the noninfectious virus present in the preparation prior to SDS treatment. It is obvious that the pleomorphic virus differs physically (in appearance) from the spherical virus, and its susceptibility to SDS suggests that it differs chemically too. The difference most probably lies in the lipid composition of the virus, since vitamin A induced pleomorphic virus has a lipid composition different from that of 'normal' virus (Blough et al.,1967) and virus grown in different hosts varies in susceptibility to SDS (Burnet and Lush, 1940); furthermore, virus grown in different tissues of the same host varies in susceptibility to phospholipase C (Simpson and

[+]Paper 1

Hauser, 1966).

Although the ideal influenza preparation of one ID_{50} per physical virus particle has yet to be achieved, by using SDS to disrupt much of the noninfectious virus we are now able to prepare virus with a particle: ID_{50} ratio closer to the ideal than has hitherto been achieved. Thus we are now in a better position to investigate the structural chemistry, and the genetics, of the infectious virion.

PAPER 4.

The effect of cystine cross-links on the mobility of proteins in dodecyl sulphate-polyacrylamide gels.

Introduction.

Shapiro, Vinuela and Maizel (1967) reported that the rate of migration of polypeptides in polyacrylamide gels in the presence of SDS* is dependent on the molecular weight of the polypeptide. Their findings have been confirmed for a large number of different proteins of well characterised molecular weights (Shapiro and Maizel, 1969; Weber and Osborn, 1969; Dunker and Rueckert, 1969). The report by Pitt-Rivers and Impiombato (1968) that disulphide-stabilised proteins bound up to 50% more SDS by weight after reduction, suggested that unreduced proteins might behave in an anomalous fashion in SDS-polyacrylamide gels. This paper reports an investigation of this possibility. As there are few well characterised proteins containing a high content of cystine, artificial oligomers of a number of proteins were prepared to serve as models in this investigation. From the behaviour of these model polypeptides, it is concluded that, generally, the migration rate of proteins in SDS-polyacrylamide gels is significantly altered by the presence of several intrachain disulphide bonds.

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* Abbreviations: SDS, sodium dodecyl sulphate; TEMED, N,N, N',N'-tetra methylethylenediamine; BSA, bovine serum albumin.

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MATERIALS AND METHODS.

Analytical grade $NaH_2PO_4.H_2O$, $Na_2HPO_4.7H_2O$, EDTA, methanol and ammonium persulphate were obtained from British Drug Houses. Analytical grade acetic acid and NaN_3 were from Ajax Chemicals (Vic) Ltd., Melbourne. Tris base was obtained from Sigma. Acrylamide, N,N'-methylenebisacrylamide, TEMED and β-mercaptoethanol were obtained from Eastman Kodak. Dithiothreitol and iodoacetamide were obtained from Calbiochem. Sodium dodecyl sulphate (95%) was obtained from Matheson Coleman and Bell. Urea was obtained from Mallinkrodt Chemical Works, New York. Glutaraldehyde was obtained as a 25% solution from L. Light and Co., Colnbrook, Bucks. Amido black was obtained from Schmid and Co., Chroma-Gesellschaft Stuttgart-Untertukheim, Procion blue from Imperial Chemical Industries (Australia) Ltd., and bromophenol blue was obtained from G.T. Gurr Ltd., London.

The proteins used were ovalbumin, ribonuclease and lysozyme (Nutritional Biochemicals Corporation), pepsin and soybean trypsin inhibitor, (Worthington), A and B variants of β-lactoglobulin (Koch-Light Laboratories, Colnbrook, Bucks), glucagon (Lilly), Bacillus subtilis α-amylase (Type IIA, Sigma), BSA and human γ-globulin (Commonwealth Serum Laboratories, Melbourne). Human $\alpha_2\beta_2$ haemaglobin was prepared from erythrocytes; the cells were washed three times with buffered saline (0.15M NaCl in 0.01M phosphate buffer, pH 7.2) and lysed with 10 volumes of distilled water; the

5/...

haemoglobin was clarified by centrifugation for four hours at 35,000 r.p.m. in a Spinco 40 rotor and then lyophilised.

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Preparation of samples for electrophoresis.

Fifteen mg. of each of BSA, ovalbumin, α-amylase, haemoglobin β-lactoglobulin, lysozyme and ribonuclease were dissolved in 1 ml aliquots of sodium phosphate buffer (0.1M, pH 7.0) in a screw-cap vial. After addition of 2 µl of 25% glutaraldehyde to each, the samples were gently agitated at room temperature for two hours. Half of each sample was transferred to a fresh vial containing 0.05 ml 1 M-iodoacetamide; 0.05 ml 0.5 M-dithiothreitol was then added to the remaining portion of each sample. To all samples 0.25 ml 2 M-tris base, 0.25 ml 10% (w/v) SDS and 0.05 ml 5% (w/v) EDTA were added. The vials were capped and placed in cold water which was slowly brought to the boil. After treatment at 100° for two minutes, the vials were cooled and 1.0 g solid urea added to each. All samples were incubated at 35° for three hours. To the samples reduced with dithiothreitol, 0.1 ml 1 M-iodoacetamide (in a 1 M-$NaHCO_3$ solution) was then added. All samples were dialysed for 8 hours against 500 volumes distilled water (one change), and then for 16 hours against 0.01 M-sodium phosphate buffer, pH 7.2, containing 0.1% SDS, 6% (w/v) sucrose and 0.01% sodium azide to prevent bacterial growth.

Haemoglobin and ribonuclease were also reacted with glutaraldehyde in the presence of SDS. Fifteen mg of each protein were dissolved in 1.0 ml sodium phosphate buffer (0.1 M, pH 7.0). The samples were divided into two equal portions. Iodoacetamide was added to one portion and dithiothreitol to the other, and to all were

7/...

added SDS, tris, EDTA, heat, urea, and to the reduced samples iodoacetamide as described above. The samples were separately dialysed (with one change) against 100 volumes 0.1 M-sodium phosphate buffer (pH 7.0) containing 0.1% SDS for 16 hours. They were then reduced in volume to about 0.3 ml by pervaporation at room temperature. Dialysis was continued against fresh buffer overnight. The samples were then made up to 0.5 ml with dialysis buffer and reacted for 2 hours at room temperature with 1 µl of 25% glutaraldehyde. The samples were then dialysed against water followed by the sucrose buffer as described above.

Lysozyme and ribonuclease, after reduction and alkylation were also treated with glutaraldehyde in the presence of 8 M-urea. 7.5 mg portions were reduced and treated as described above, but without the addition of SDS or glutaraldehyde. After the reaction with iodoacetamide, samples were dialysed against 100 volumes 8 M-urea in 0.1 M-sodium phosphate buffer (pH 7.0) for 24 hours, with changes at 8 and 18 hours. Samples were then treated with 1 µl 25% glutaraldehyde at room temperature for two hours. Mercaptoethanol (0.025 ml) was added to all samples, which were then dialysed against the sucrose buffer described above.

Polyacrylamide gels.

Polyacrylamide gels were prepared according to Summers, Maizel and Darnell (1965). 10 - 12 cm gels were cast in 14 cm glass tubes (with an internal diameter of 6 mm), mounted vertically on

Parafilm. Gel and electrophoresis buffers were 0.1 M-sodium phosphate, pH 7.2, containing 0.1% SDS and 0.01% sodium azide. The gels were 5% (w/v) with respect to acrylamide, 0.13% with respect to N,N'-methylene bisacrylamide, and were polymerised with 0.1% ammonium persulphate and 0.05% (v/v) TEMED. Gels were overlayered with distilled water. After polymerisation, the water was replaced with electrophoresis buffer, and the gels stored at room temperature before use.

Electrophoresis.

Protein samples were mixed with a few µl of glucagon solution (5 mg in 1 ml 1% (w/v) SDS) which was used as an internal reference marker, and a trace of bromophenol blue (which was used as a running guide). Between 5 and 35 µl of each sample containing 15 - 150 µg protein were layered under the buffer upon the surface of the gel. Electrophoresis was carried out at constant current for various times (usually 9 mamp per tube for 4 hours).

Staining.

After electrophoresis, gels were stained by agitating for 1 hour in acetic acid: methanol: water, 1:4:5 (by volume) containing 1% (w/v) Procion blue and 2% (w/v) Amido black. Gels were destained by agitation in acetic acid: methanol: water, 1:8:10 (by volume) overnight, followed by 7% (v/v) acetic acid for 24 hours. Gels were photographed through a Wratten 23A filter.

9/...

Measurement of Relative Mobilities.

The distance from the discontinuity at the top of the gel (visible as a refractile band, or as a change in degree of background stain) to the centre of each protein band was measured. The values were converted to relative mobilities by dividing each by the distance migrated by the glucagon marker.

10/...

RESULTS

Since it was intended to compare small differences in mobility, it was first necessary to ensure that the mobility of proteins relative to a given standard were constant. The internal standard chosen was glucagon. Nine proteins were chosen to construct a standard curve. Fig. 1 shows a plot of mobility relative to glucagon versus the molecular weights given in Table I. Each point represents the mean of a number of experiments (indicated in parenthesis for each protein in Fig. 1), and the 99% confidence limits are shown. The line was fitted to the mean values for each protein by the method of least squares using an IBM 7044 computer. The values for ribonuclease and lysozyme were not used for this standard curve, as these proteins are reported (Shapiro et al, 1967; Dunker and Rueckert, 1969) to behave anomalously in SDS-polyacrylamide gels. It can be seen that the mobilities measured relative to glucagon were highly reproducible between experiments, provided that measurements were taken from the boundary of the gel proper and the visible discontinuity, present at the top of the gel. This discontinuity, which varies from gel to gel, is probably the greatest source of error, especially for proteins of low mobility (Maizel, 1969). Fig. 1 also shows that deviations from the expected curve are reproducible (e.g. compare γ-globulin L chain and soybean trypsin inhibitor). Fig. 2 shows the polypeptide patterns obtained after reacting a number of proteins with glutaraldehyde and denaturing them with and without simultaneous reduction. The band of highest mobility in

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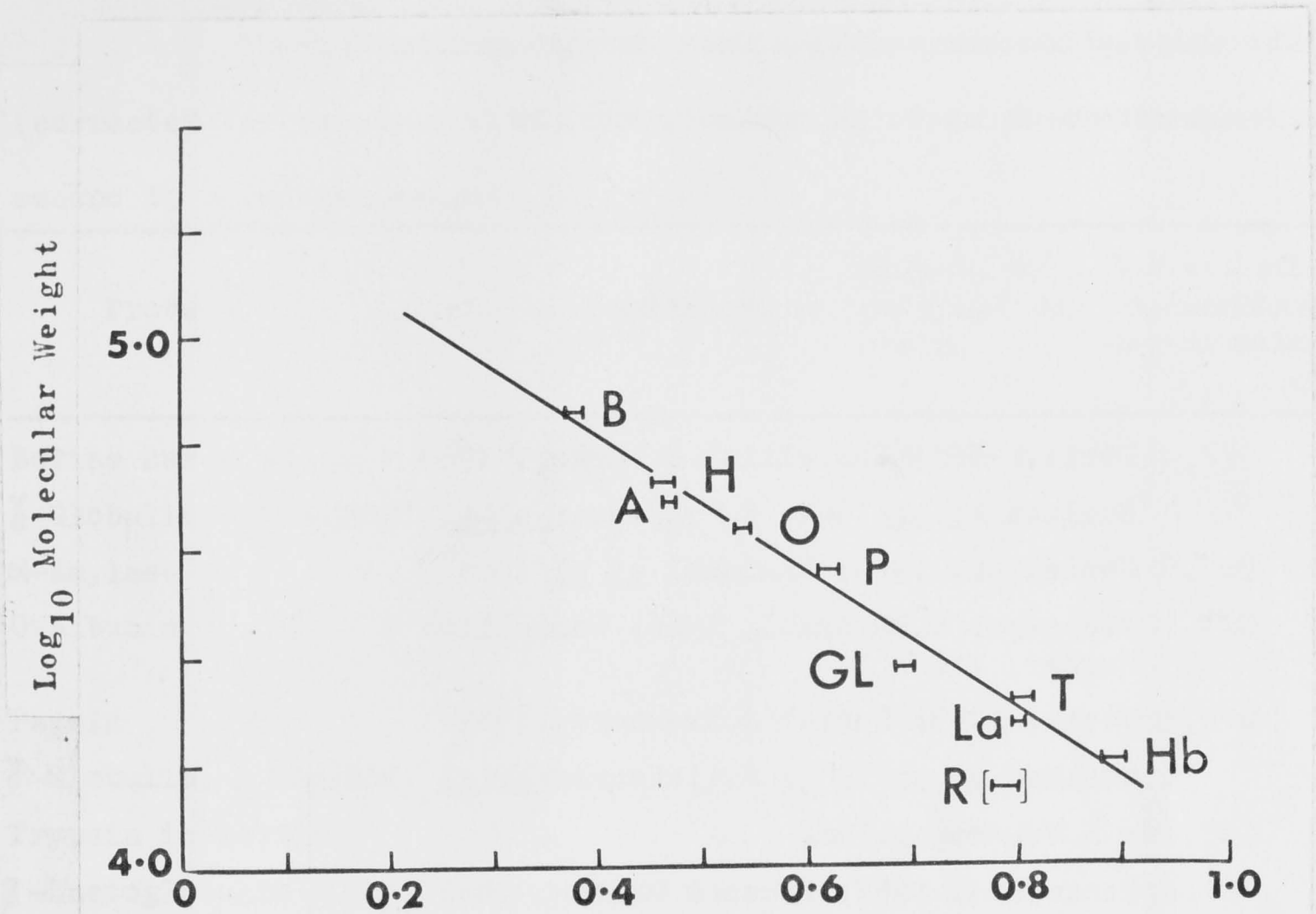

Fig. 1. Plot of relative mobility versus $\log_{10}$ molecular weight of the protein. Each point represents the mean of a number of measurements (given below in parentheses for each protein) and the 99% confidence limits are indicated. The line of best fit for the mean values (excluding ribonuclease, R) was determined by the method of least squares. B = bovine serum albumin (34), H = γ-globulin heavy chain (10), A = α-amylase (13), O = ovalbumin (11), P = pepsin (13), GL = γ-globulin light chain (10), T = trypsin inhibitor (11), La = β-lactoglobulin (13), Hb = haemoglobin (11), R = ribonuclease (10).

TABLE I. Proteins studied by SDS-electrophoresis.

The table lists cysteine and cystine content and molecular weights of the polypeptide chains taken from the literature (corrected for reaction with iodoacetamide). Where two references are cited, the first refers to cysteine content, and the second to molecular weight.

Protein	Cysteine	1/2 Cystine	Mol.wt. of polypeptide chain	Mol.wt. of iodoacetamide derivative *	Mol.wt. of reduced protein iodoacetamide derivative	Reference
Bovine serum albumin	1 ***	43	68,530 ø	68,590	70,530	Cecil, 1963; Castellino & Barker, 1968
γ-Globulin, H chain	11 øø		51,600		52,230	Edelman et al 1969; Rutishauser et al 1969
α-Amylase	0	0	48,200	48,200	48,200	Connellan, 1968; Kakiuchi et al 1965
Ovalbumin	5	2	43,830 ø	44,120	44,220	Neuberger & Marshall, 1966; Castellino & Barker, 1968
Pepsin	0	6	35,500	35,500	35,840	Cecil, 1963; Dunker & Rueckert, 1969
γ-Globulin, L chain	5 øø		23,500			Edelman et al 1969; Rutishauser et al 1969
Trypsin inhibitor	0	4	21,500	21,500	21,730	Wu & Scheraga, 1962
β-Lactoglobulin	1	4	18,400	18,460	18,680	Mackenzie, 1967; Weber & Osborn, 1969
Haemoglobin	1.5 øøø	0	15,500øøø	15,585	15,585	Weber & Osborn, 1969
Lysozyme	0	8	14,310	14,310	14,760	Canfield & Liu, 1965; Canfield, 1963
Ribonuclease	0	8	13,700	13,700	14,160	Smyth et al 1963; Weber & Osborn, 1969
Glucagon	0	0	3,500	3,500	3,500	Behrens & Bromer, 1968

* Protein denaturated in the presence of iodoacetamide

** Protein denaturated in the presence of dithiothreitol, and later reacted with iodoacetamide

*** After reduction of undenaturated protein to mercaptalbumin (Cecil, 1963)

ø The mean of the values for the native proteins determined by Castellino and Barker (1968).

øø After reduction of denaturated protein.

øøø The mean value for a mixture of equal amounts of α and β chains.

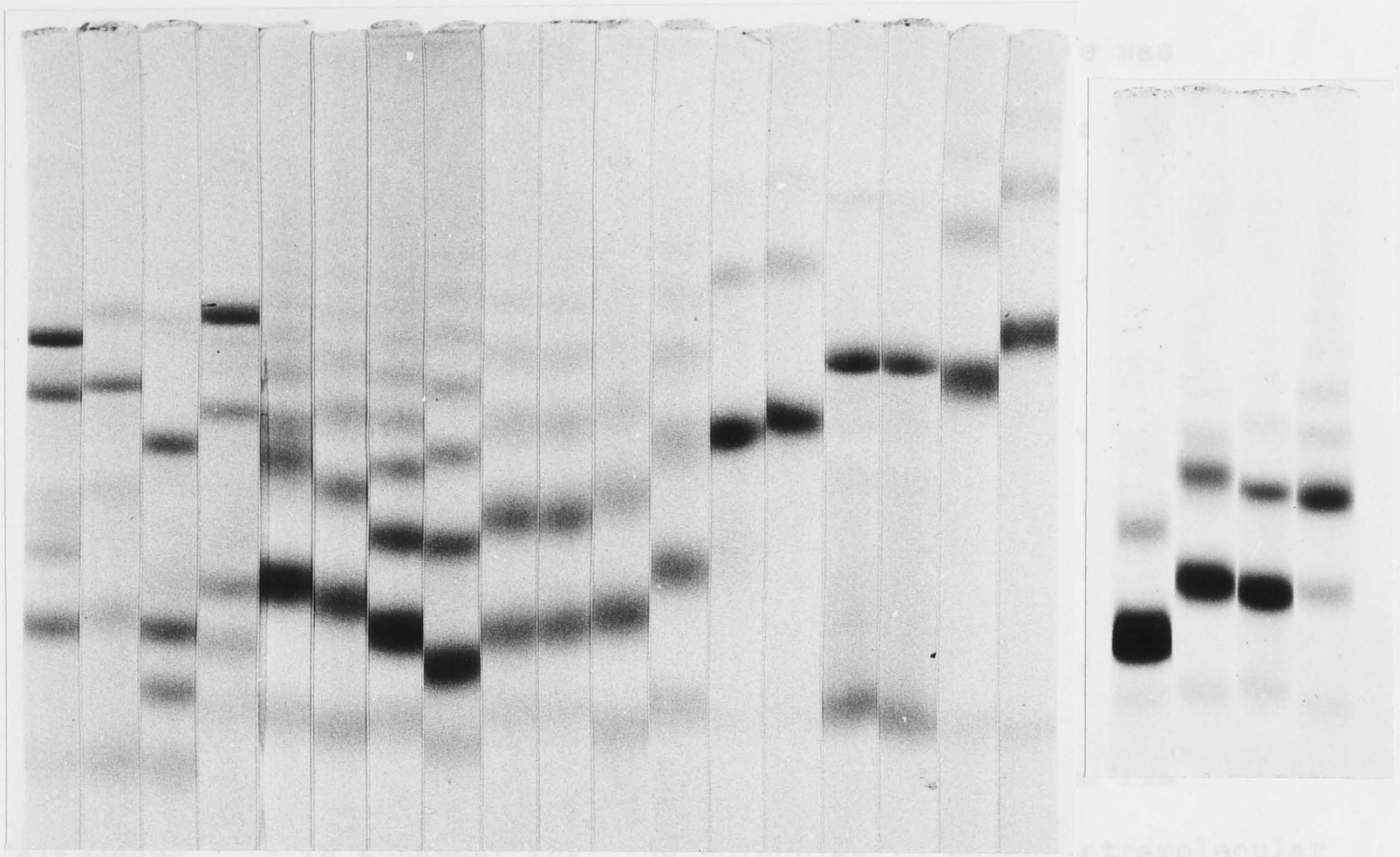

Fig. 2. Analytical polyacrylamide gels of standard proteins (a - d) and proteins reacted with glutaraldehyde before denaturation (e - r) and after denaturation (s - v). In all gels the direction of migration was from top to bottom; the band of highest mobility was glucagon. (a) Mixture of BSA, γ-globulin and ribonuclease; (b) Mixture of BSA, α-amylase, pepsin and β-lactoglobulin; (c,d) Mixture of BSA, ovalbumin, tryspin inhibitor and haemoglobin. In each of the following gels the protein pairs were (unreduced sample first): ribonuclease (e,f), lysozyme (g,h), haemoglobin (i,j), β-lactoglobulin (k,l), ovalbumin (m,n), α-amylase, (o,p), BSA (q,r). Haemoglobin (s) reacted with glutaraldehyde after denaturation without reduction; ribonuclease (t,u) denatured in SDS without reduction, reacted with glutaraldehyde and then electrophoresed without (t) or with (u) prior reduction; ribonuclease (v) denatured, carboxymethylated and then reacted with glutaraldehyde in the presence of SDS.

each case was glucagon. The mobility of each polypeptide was measured relative to that of glucagon and the mean values (obtained from four to six gels) plotted against the $\log_{10}$ of the molecular weights (Fig. 3). In calculating the molecular weights of the polypeptide oligomers from the values given in Table 1, it was assumed that cysteine residues, either indigenous or formed on reduction, had all reacted with iodoacetamide, and that only n-1 glutaraldehyde molecules were bound per oligomer (where "n" is the number of polypeptides assumed to be present in each oligomer).

Fig. 3a shows the plots for β-lactoglobulin and its oligomers. It is apparent that the molecules in which intramolecular disulphide bridges have been destroyed migrate more slowly than their unreduced counterparts. The differences were significant at the 5% level, as were all other comparisons in Fig. 3, unless otherwise stated. The mobilities for ovalbumin oligomers, both reduced and unreduced after reaction with glutaraldehyde, are also shown in Fig. 3a; the differences in these cases were not significant. Also shown are the mobilities of denatured ovalbumin which had been reacted with neither glutaraldehyde nor a reducing agent. The mobility of the monomer of this is significantly different from both that of the glutaraldehyde treated but unreduced ovalbumin and that of the reduced ovalbumin used for the standard curve. In contrast neither the mobilities of the various dimers nor those of the various trimers of ovalbumin differed significantly.

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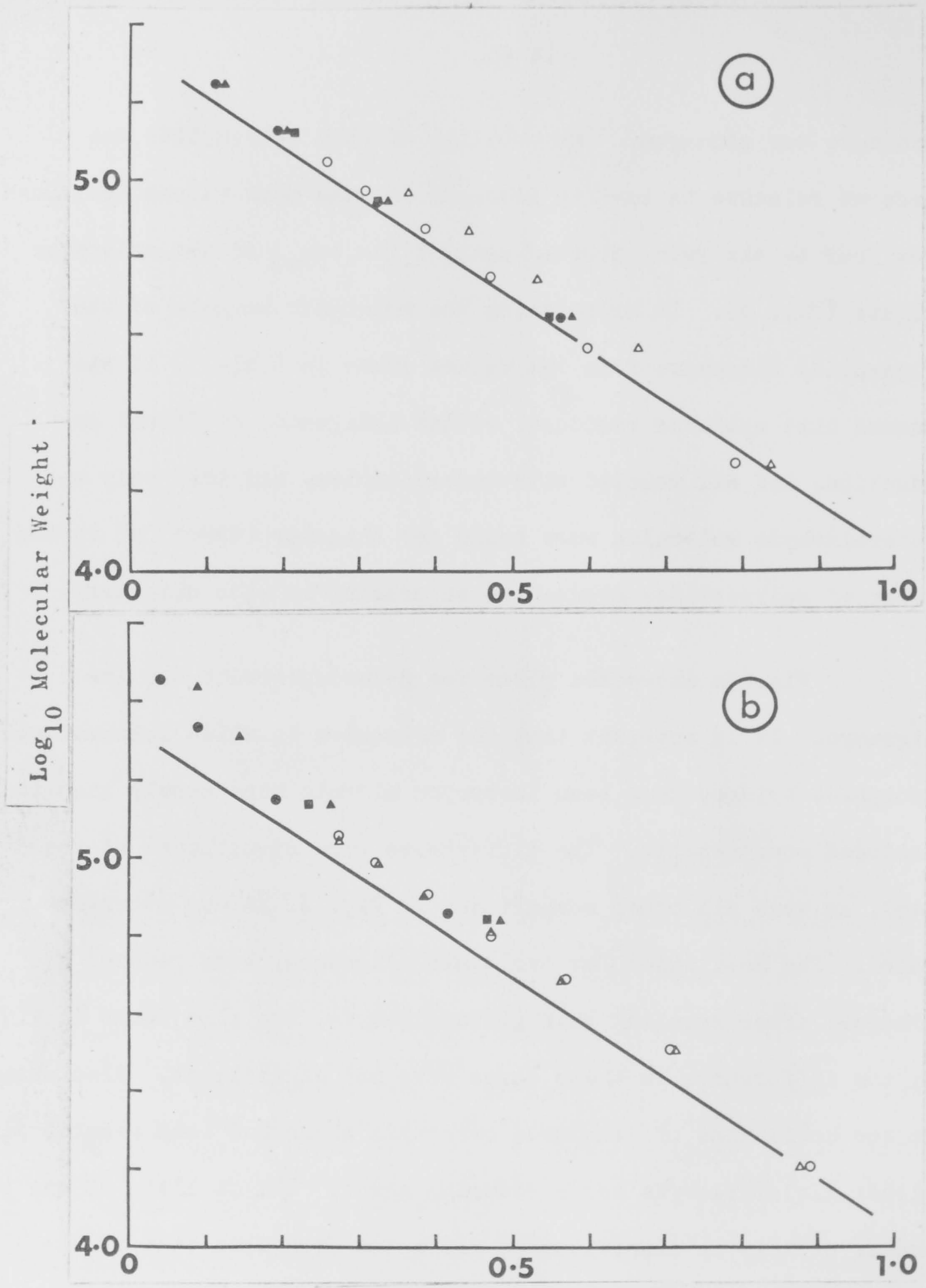

Fig. 3. Plots of $\log_{10}$ molecular weight versus relative mobility for various proteins treated with glutaraldehyde after denaturation with reduction (squares, Fig. 3.c,d), denatured without reduction or reaction with glutaraldehyde (squares, Fig. 3.a,b), proteins treated with glutaraldehyde before denaturation with reduction (circles, Fig. 3.a - d) and before denaturation without reduction (triangles, Fig. 3a - d).

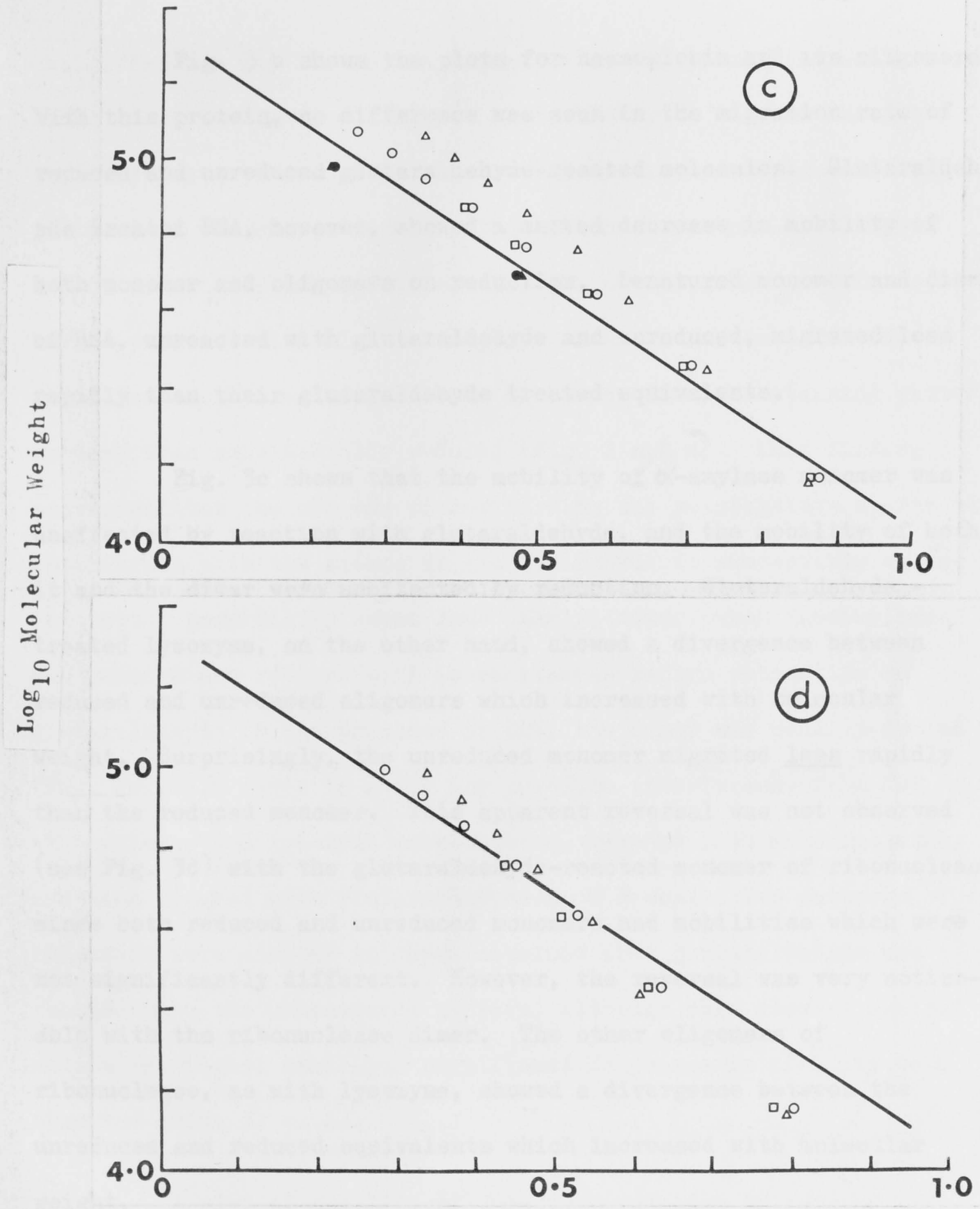

Fig. 3 (con't). (a) open points = β-lactoglobulin; filled points = ovalbumin. (b) open points = haemoglobin; filled points = BSA. (c) open points = lysozyme; filled points = ∝-amylase. (d) open points = ribonuclease.

The continuous line represents the standard curve plotted in Fig. 1.

Fig. 3 b shows the plots for haemoglobin and its oligomers. With this protein, no difference was seen in the migration rate of reduced and unreduced glutaraldehyde-reacted molecules. Glutaraldehyde treated BSA, however, showed a marked decrease in mobility of both monomer and oligomers on reduction. Denatured monomer and dimer of BSA, unreacted with glutaraldehyde and unreduced, migrated less rapidly than their glutaraldehyde treated equivalents.

Fig. 3c shows that the mobility of α-amylase monomer was unaffected by reaction with glutaraldehyde, and the mobility of both it and the dimer were unaffected by reduction. Glutaraldehyde - treated lysozyme, on the other hand, showed a divergence between reduced and unreduced oligomers which increased with molecular weight. Surprisingly, the unreduced monomer migrated less rapidly than the reduced monomer. This apparent reversal was not observed (see Fig. 3d) with the glutaraldehyde-reacted monomer of ribonuclease, since both reduced and unreduced monomers had mobilities which were not significantly different. However, the reversal was very noticeable with the ribonuclease dimer. The other oligomers of ribonuclease, as with lysozyme, showed a divergence between the unreduced and reduced equivalents which increased with molecular weight.

Generally, the reduced glutaraldehyde-reacted polypeptides migrated more rapidly than one might have expected from the standard curve. Since the presence of intramolecular disulphide links appeared to result in increased mobility, a possible explanation for

13/...

the difference may lie in the formation of additional intramolecular cross links during reaction with glutaraldehyde. It was thought that unfolding of the protein before glutaraldehyde treatment might reduce this tendency. Consequently, ribonuclease and haemoglobin were treated with SDS and iodoacetamide, as described in Methods, before reaction with glutaraldehyde. Although not quantitated, formation of intermolecular bonds appeared, from the staining patterns, to have been substantially reduced (Fig. 2 s, t, w). This finding suggested that the SDS was either holding the polypeptides apart, or interfering with the access of glutaraldehyde to susceptible sites. The latter possibility seems less likely, since, when ribonuclease was reduced and reacted with iodoacetamide before subjection to glutaraldehyde in the presence of SDS, the dimer was readily formed (Fig. 2v). In order to avoid any possible interference from SDS, ribonuclease and lysozyme were reduced, reacted with iodoacetamide, and then reached with glutaraldehyde in 8 M-urea. The patterns of oligomers were similar to those obtained when glutaraldehyde was reached with the undenatured protein, although when plotted against molecular weight, small but significant decreases in mobility were observed (Fig. 3, c, d).

A plot of mobility of unreduced relative to reduced polypeptide versus cystine content might reveal whether any relationship exists between change in mobility and cystine content. Fig. 4 shows that this is the case, and that, although the plots obtained for the

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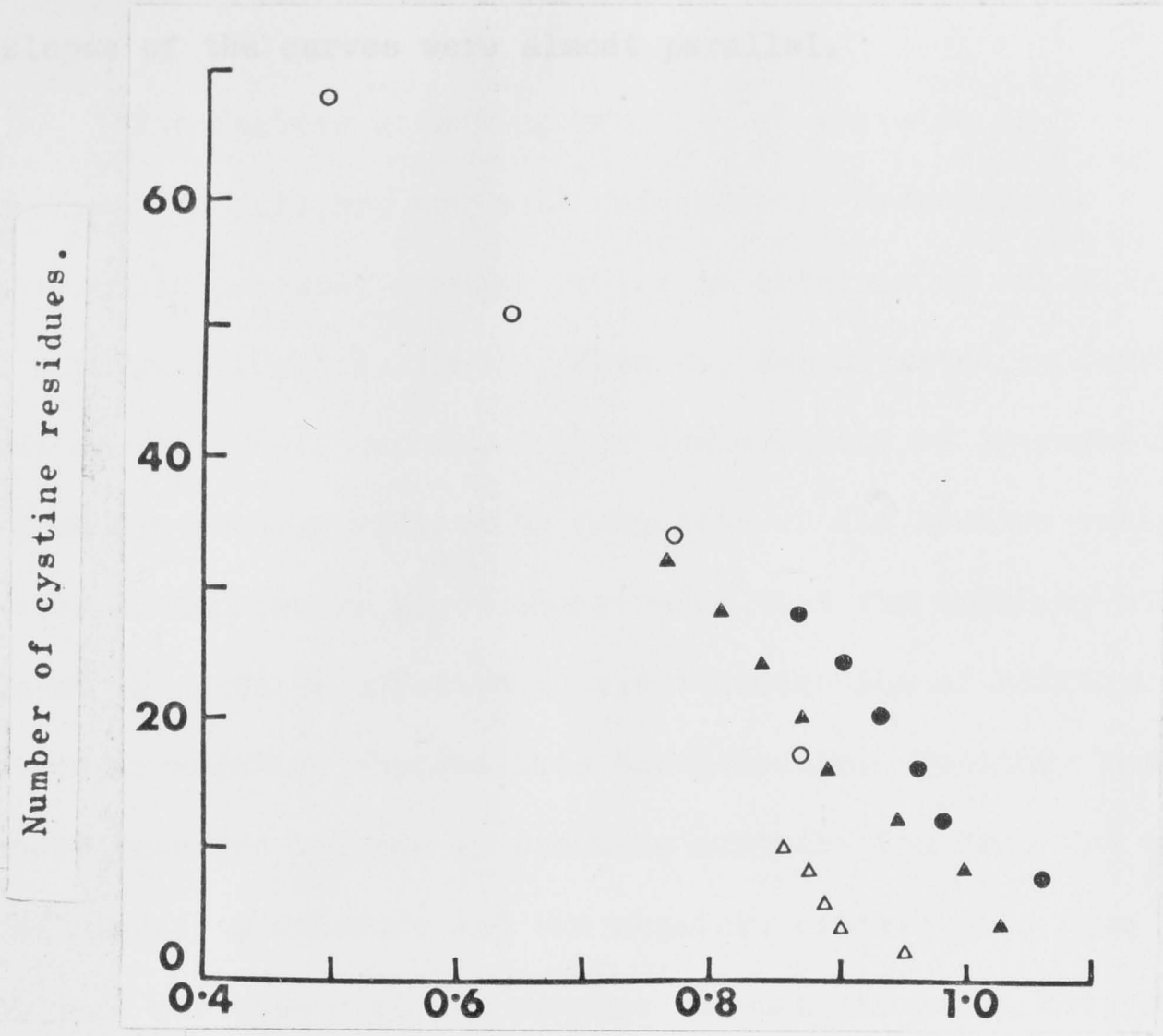


Fig. 4. Plot of cystine content of protein oligomers versus the ratios of the mobilities of the unreduced to the reduced protein oligomer. Open circles = BSA; filled circles = ribonuclease; open triangles = β-lactoglobulin; filled triangles = lysozyme.

four proteins differed, for the higher molecular weight oligomers the slopes of the curves were almost parallel.

DISCUSSION

The factors affecting mobility of proteins in polyacrylamide gels are not well understood. Hydrodynamic resistance is probably a major factor in determining mobility in fine pore gels (Ogston, 1966). This was demonstrated by Dunker and Rueckert (1969), who found that the mobility of lysozyme in 8 M-urea was greatly reduced by reduction of its cystine residues. However, these workers also demonstrated that the mobility of lysozyme was greatly affected by the introduction of additional positive or negative charges into the molecule. Mobility thus depended upon the balance of positive contribution from the charge of the migrating molecule and the negative contribution from the frictional resistance to its passage through the gel. The relative contribution of these two parameters is difficult to ascertain where the migrating species is a polypeptide-SDS complex; the larger the polypeptide, the more dodecyl sulphate it will bind, and hence both the size of the SDS complex and its negative charge will be increased. Where the increase in mobility arising from increased negative charge offsets the decrease in mobility due to an increase in size of the SDS-polypeptide complex, we might expect that variations in the affinity of different proteins for dodecyl sulphate would have little effect on the mobility of SDS-polypeptide complexes in polyacrylamide gels. This explanation, tendered by Duncker and Rueckert (1969), probably accounts for the barely

16/...

significant changes in mobility in SDS-polyacrylamide gels accompanying chemical alteration of the charge of polypeptides (Shapiro and Maizel, 1969; Weber and Osborn, 1969; Dunker and Rueckert, 1969) or the chain unfolding consequent upon reduction (Dunker and Rueckert, 1969).

It was considered necessary to examine a large number of different polypeptides containing variable numbers of cystine residues in an effort to determine what effect the unfolding of cystine-stabilised polypeptides would have on their mobility in SDS-polyacrylamide gels. Only a few well characterised proteins with many cystine residues are at present available, and for this reason model polypeptides were prepared by cross-linking proteins with glutaraldehyde. Glutaraldehyde is able to react with protein lysine residues (Quicho and Richards, 1966). As it is a bifunctional reagent, it may react with two residues. Where these are on different proteins, dimers are formed, and these may react further giving trimers, tetramers, etc; this is presumably the cause of the higher molecular weight oligomers present in the glutaraldehyde-treated proteins described in this paper. Intramolecular cross-links may also form within proteins during reaction with glutaraldehyde (Bowes and Cater, 1965). However, since the unreduced glutaraldehyde-linked proteins differ from their reduced equivalents only in the possession of cystine residues, we must conclude that the observed difference in mobilities of each

17/...

corresponding pair of oligomers must be a consequence of their different tertiary structure. Fig. 4 shows that there is an almost linear relationship between the number of cystine residues and the decrease in mobility, although (with the exception of β-lactoglobulin) this decrease was small where few cross-links were present. The finding that the unreduced BSA trimer migrated at about half the rate of the reduced trimer is particularly significant in view of the use of oligomers of this protein as standards for molecular weight determination (Dunker and Rueckert, 1969). Fig. 4 also demonstrates that, for a given cystine content, the mobilities of different proteins are not reduced to the same extent. This is not surprising, since the degree of unfolding accompanying hot SDS and urea denaturation will depend on the position (as well as the number) of disulphide cross-links.

To what extent additional intramolecular glutaraldehyde cross-links were also present in the treated proteins was not established. Their presence in such proteins would (by analogy with the cystine cross-linked proteins) presumably lead to an increased mobility compared with the proteins of the standard curve which were completely unfolded. This would explain the greater mobility (compared with the standard curve) of most of the fully reduced glutaraldehyde-reacted polypeptides. In contrast, the mobilities of ovalbumin and α-amylase were little affected by reaction with glutaraldehyde, and these proteins presumably had

few or no intramolecular glutaraldehyde cross-links. The haemoglobin oligomers were seen to migrate in the gel as brown bands. Thus, although the haem and globin were separated by the denaturation procedure before reaction with glutaraldehyde, after the reaction this was not so, presumably because of intramolecular glutaraldehyde cross-links. It was hoped that the formation of intramolecular cross-links might be eliminated by reacting alkylated ribonuclease and lysozyme with glutaraldehyde in 8 M-urea. The oligomers formed under these conditions did have a slightly lower mobility than the other reduced oligomers of these proteins, but the failure to approach the mobility of the standard proteins indicated either that some intramolecular cross-links were still present, or that the branched nature of these proteins was affecting their mobility; alternatively, the basic nature of these proteins might have affected their mobility in the SDS polyacrylamide gels.

For a given protein oligomer one might expect variation in the number of intramolecular glutaraldehyde cross-links to result in many overlapping polypeptide bands, since the presence of intramolecular disulphide bridges so markedly affect the migration rate. That a smear of polypeptides was not obtained is a little surprising, and indicated that each oligomer species contained polypeptides of approximately equivalent tertiary structure. Further variation in their tertiary structure may have arisen where, for a

given oligomer, cross-linking involved many different amino acids in the polypeptide chain; this would give branched polypeptides with branch lengths dependent on the particular amino acid residues involved. These factors probably explain why, in addition to migrating more rapidly, the polypeptide species of the glutaraldehyde-reacted proteins generally migrated as broader bands than their unreacted counterparts.

The result of Dunker and Rueckert (1969) for BSA, ovalbumin, chymotrypsinogen A and ribonuclease indicate that the folded molecule migrates a little more rapidly than the unfolded polypeptide, while the migration of lysozyme was unaffected by unfolding. The results reported here indicate that the mobility of ribonuclease monomer is unaffected by the presence of cystine bridges, whereas the mobility of lysozyme monomer is <u>increased</u> by chain unfolding (as is also that of the ribonuclease dimer). It is not clear from the work of Dunker and Rueckert what polyacrylamide gel concentration was used in their experiment or how repeatable their observations were. In any case, the results are not strictly comparable since glutaraldehyde-treated proteins have been used in this work. However, what is clear, from both their work and that reported here, is that certain low molecular weight proteins behave in an anomalous fashion in 5% SDS-polyacrylamide gels.

The anomalous behaviour of ribonuclease and lysozyme

20/...

(Shapiro et al., 1967) and other low molecular weight proteins (Dunker and Rueckert, 1969) has been ascribed (Dunker and Rueckert, 1969) to rapid, reversible dimerisation in solution. The reaction of reduced and carboxymethylated ribonuclease with glutaraldehyde in the presence of SDS supports this proposal, as the increased formation of dimer suggests that two polypeptides are held in close contact by the detergent during the reaction with glutaraldehyde. Pitt-Rivers and Impiombato (1968) have estimated that the apparent micellar molecular weight of SDS complexes with protein to be in the range of 34,000 - 40,000 daltons, and estimated that the amount of SDS bound to reduced protein in 66 mM-sodium phosphate buffer (pH 7.2) to be about 1.4 mg per mg protein; this was reduced to about 0.4 mg SDS per mg protein on the addition of 0.154 M sodium chloride. While the amount of SDS bound per mg protein in 0.1 M sodium phosphate buffer, pH 7.2, is not known, it is probably much less than 1.4 mg SDS per mg protein (and may, in fact, be estimated to be 0.2 mg SDS per mg protein, within the gel matrix of the polyacrylamide gel; Shapiro and Maizel, 1969). We might expect SDS-saturated ribonuclease to be below the minimum micellar molecular weight, the latter being reached by incorporating two polypeptide molecules into the micelles. Such a complex cannot be stable, since the apparent molecular weight of the reduced ribonuclease is not doubled but merely increased to about 20,000 daltons (Fig.1.). Shapiro and Maizel (1969) have shown that protein-bound SDS is

21/...

freely exchanged with buffer SDS during electrophoresis, and so it seems likely that an equilibrium

$$a\text{SDS} + n\text{Ribonuclease} \rightleftharpoons \text{SDS}^{a-b}\text{:Ribonuclease}^{n} + b\text{SDS}$$

exists where excess SDS is present; the position of equilibrium, and the value of n under the conditions of the experiment would dictate the degree of retardation observed. The expected relative mobility value for ribonuclease from Fig. 1 is 0.92 and the observed value 0.78. One may estimate that during electrophoresis, on average, the SDS-ribonuclease complex exists as an aggregate of two polypeptides 15% of the time (where n is assumed to be two). However, the value of n may well be greater than two, and indeed the behaviour of glutaraldehyde-linked ribonuclease suggests that for this protein at least, the polypeptide approaches the behaviour of the standard proteins only when n is equal to five. Lysozyme also showed a similar trend, which is less obvious for the reduced protein than for the unreduced protein. Since the plot of mobility against log-molecular weight for the higher oligomers of these two proteins approximates to linearity, their anomalous behaviour cannot be ascribed to the amino acid composition per se, but to a combination of size and composition which affects the manner in which they interact with SDS. Pitt-Rivers and Impiombato (1968) suggested that micelles of SDS bind as beads along the polypeptide backbone of proteins of molecular weight exceeding 20,000 daltons. They found that when SDS was bound it was present as micelles, even if the

22/...

polypeptides (such as those of polylysyl-glutamic acid or glycoproteins) bound less SDS than more 'normal' proteins, and they suggest that, in these atypical proteins, the number of specific detergent-binding sites is reduced. On this model one might expect that the highly cross-linked proteins contain a number of binding sites different from their unfolded counterparts. However, we do not know whether SDS is bound as micelles by the polypeptides as they pass through the fine pores of polyacrylamide gels. Indeed the low level of SDS bound by the migrating polypeptide (as demonstrated by Shapiro and Maizel, 1969) may indicate that some other form of interaction occurs during gel electrophoresis.

The demonstration in this paper that a decrease in mobility in SDS-polyacrylamide gels accompanies unfolding of the polypeptide, and that certain polypeptides migrate in an anomalous fashion in these gels, emphasises that molecular weights estimated by the SDS-polyacrylamide gel method of Shapiro et al. (1967) may on occasions be misleading. This applies especially to proteins or glyco-proteins which resist the denaturation procedures employed by these workers; the haemagglutinin of influenza virus is an example of such a protein (I.P. Griffith, in preparation[+]). Furthermore, the use of unreduced BSA or γ-globulin, and their cystine cross-linked oligomers, in the preparation of standard curves for molecular weight determination (Dunker and Rueckert, 1969) may give too high an estimate for the molecular weight of the unknown polypeptide under investigation.

[+]Paper 5

23/...

PAPER 5.

The polypeptides of the influenza virion.

Influenza virus contains three known proteins - the haemagglutinin (HA), the neuraminidase and the ribonucleoprotein (RNP). The 'host-antigen' component is thought to be carbohydrate covalently bonded to the viral protein (Griffith, 1969). The polypeptides of denatured, purified virions have been analysed in SDS-polyacrylamide gels by a number of workers (Duesberg and Robinson, 1967; Griffith, 1968, 1969; Skehel and Burke, 1969; Joss et al., 1969; Taylor et al., 1969; Pons and Hirst, 1969; Pons et al., 1969; Dimmock and Watson, 1969; White et al., 1970). The number of major polypeptides varies from three (Duesberg and Robinson, 1967; Griffith, 1968, 1969; Taylor et al., 1969) to several (Dimmock and Watson, 1969). Some workers claim the largest polypeptide is derived from the haemagglutinin (Griffith, 1968; Taylor et al., 1969; White et al., 1970), while others suggest it is the RNP (Pons et al., 1969; Joss et al., 1969).

The systems used for polyacrylamide gel analysis, the virus strains investigated and the host cell in which they have been grown, and the denaturation procedures used to reduce the virion to polypeptides have been different in different laboratories. This paper describes an investigation into what way different denaturation procedures affect the polypeptide pattern in analytical polyacrylamide gels, how this pattern is affected by acrylamide concentration, and whether different virus strains grown in eggs show differences in polypeptide composition.

MATERIALS AND METHODS.

Chemicals.

EDTA, Na_2HPO_4, $NaHPO_4$, $NaHCO_3$, Na_2CO_3, hydrogen peroxide and formic acid (all A.R.) and guanidine hydrochloride were obtained from British Drug Houses; formic acid was redistilled. Urea was obtained from Mallinkrodt; SDS from Matheson, Coleman and Bell; NP 40 from Shell (Australia) Ltd. Dithiothreitol (DTT) and iodoacetamide were from Calbiochem; β-mercaptoethanol, acrylamide, 'bis'acrylamide and 'TEMED' were obtained from Eastman Kodak. Tris base was from Sigma. Buffers used were sodium phosphate, pH 7.2, pH 8.0; NaH_2PO_4-Na_2CO_3, pH 9.0; $NaHCO_3$-Na_2CO_3, pH 10.0, pH 10.7.

Virus.

The strains of influenza virus used were from stocks held at the Commonwealth Serum Laboratories, Melbourne. They were: SW (strain 15 of Shope, 1931); the BEL strain of A_0 influenza; the Asian strains A_2/RI/5$^-$, A_2/SA/64, A_2/NT/68; the A_0/A_2 recombinant $X7F_1$ of Kilbourne (Kilbourne et al., 1967); and the B/Vic/65 strain of influenza B.

Propagation and purification of virus.

Virus was grown from seed (about 10^4 ID_{50}) introduced into the allantoic cavity of 10 - 11 day old fertile chicken eggs. After incubation at 33° for about 40 hours, eggs were chilled and the allantoic fluid harvested. The BEL strain was concentrated by precipitation with 40% (w/v) ammonium sulphate, and purified by

fractional precipitation with methanol (25 - 35% v/v methanol fraction). Other strains were purified and concentrated by two cycles of adsorption-elution on washed human erythocytes. All strains were further purified by differential, rate-zonal and buoyant -density centrifugation as described elsewhere (Griffith, in preparation[+]).

Preparation of haemagglutinin of BEL.

The procedure of Laver (1964) was used except that SDS-treated BEL was sonicated before electrophoresis (1 min., maximum output, Mullard ultrasonic drill) to assist disruption of the virus.

Denaturation of virus.

1) Performic acid oxidation.

Virus samples (about 10 mg) were freeze-dried and oxidised with excess performic acid for 2 or 3 hours at 0° as described by Hirs (1956). At the end of this time, excess performic acid was destroyed by adding 0.3 ml β-mercaptoethanol.

2) High pH, high temperature denaturation.

Virus samples (about 5 mg in 0.25 ml physiological saline) were mixed in screw cap vials with 0.1 ml SDS (10%, w/v, aqueous solution), 0.25 ml 1 M buffer of appropriate pH or 0.25 ml 2 M-tris base, 0.1 ml EDTA (5%, w/v), 0.1 ml 0.5 M-dithiothreitol (DTT) or 0.025 ml of a warm (37°) solution of 1 M-iodoacetamide in 1M-$NaHCO_3$. Vials were placed in a beaker of water which was brought to the boil and held at 100° for 5 minutes. Samples were placed at 37° for

[+]Chapter 3

2 hours. Solid urea was added to give a saturated solution, and samples were held at 37° for a further 2 hours. Samples reduced with DTT were carboxymethylated by the addition of 0.125 ml of the 1 M-iodoacetamide solution. After 15 minutes, 0.05 ml of β-mercaptoethanol was added to react with any excess iodoacetamide.

3) NP 40 - guanidine hydrochloride - urea - DTT denaturation.

Freeze-dried samples were dissolved in 0.3 ml NP 40 (10% v/v in water), 0.20 ml 1 M-$NaHCO_3$, 0.2 ml 0.5 M-DTT, 0.1 ml 5% (w/v) EDTA. Solid guanidine hydrochloride was added until the solution was saturated; solid urea was then added until the solution was saturated with urea; further cycles of addition of solid guadinine hydrochloride followed by solid urea were then made until the solution was saturated with both salts. Samples were left for 5 hours at room temperature, and then transferred to dialysis tubing, together with a distilled water rinse (0.3 ml) of the glass vials. Samples were dialysed for 16 hours against 8 M-urea in 0.01 M-$NaHCO_3$, 0.1% NP 40 and 0.2% β-mercaptoethanol to remove the guanidine hydrochloride, followed by dialysis against a fresh urea-$NaHCO_3$ solution containing 0.1% SDS (w/v) in place of NP 40, and 0.05% (v/v) β-mercaptoethanol. Samples were reacted with 0.2 ml iodoacetamide (1 M in 1 M-$NaHCO_3$) to block exposed SH groups.

Acrylamide gel electrophoresis.

5% gels: The procedure of Dunker and Rueckert (1969) was used.

10% gels: The procedure of Weber and Osborn (1969) was used.

Samples were dialysed overnight against 0.1% SDS, 0.01 M-phosphate

buffer, 6% (w/v) sucrose, and before application to the gels, mixed with a few µg glucagon which had been pre-stained with Remazol Brilliant Blue R (Griffith, in preparation[+])

Staining of gels.

For protein: Gels were stained with 0.5% Coomassie blue in acetic acid:methanol:water (2:4:5, by volume) and destained with acetic acid:methanol:water (1:9:10, by volume).

For carbohydrate: Gels were freed of SDS by fixation in acetic acid: methanol:water (1:9:10, by volume; 24 hours with two changes) and stained for carbohydrate using the procedure of Zacharius et al. (1969).

Molecular weight determinations.

Virus samples were co-electrophoresed with prestained glucagon. Mobilities relative to the glucagon marker were calculated and molecular weights determined from a standard curve drawn from 9 proteins of known molecular weight (Griffith, in preparation[++]).

[+] Paper 2

[++] Paper 4

RESULTS.

Analysis in 5% polyacrylamide gels.

The polypeptide patterns obtained in 5% polyacrylamide gels with a number of influenza virus strains denatured in different ways are shown in Fig. 1. It is evident that the denaturation procedure used markedly affected the pattern obtained. Thus, when SW and BEL virus were denatured at elevated pH and temperature (0.25 M-tris base, 100° for 5 minutes) without simultaneous reduction of cystine residues, a number of high molecular weight polypeptides were present (bands a-e, Fig. 1e, g). If the denaturation was accompanied by simultaneous reduction these high molecular weight polypeptides were absent. In addition, the amount of the largest major polypeptide (1A) was substantially reduced, while the polypeptide 1B was increased in amount. At the same time, an additional polypeptide 1D appeared moving close behind band 3. This was present in the gels of SW, BEL, X7F_1 and $A_2$64, but not in that of the NT68 strain, nor in samples of virus oxidised with performic acid (RI/5$^-$, BEL) (see Fig.1). The gels were stained for glycoprotein and only bands 2, 1c and 3 appeared free of carbohydrate; the low molecular weight polypeptides were only poorly stained. Material staining with the periodic acid-Schiff stain was also present in, or in front of, the glucagon band; this was probably non-dialysable complex lipid.

On paper electrophoresis of SDS-disrupted BEL, two protein bands with haemagglutinating activity are present (Laver, 1964). That closest to the origin, was found after denaturation with reduc-

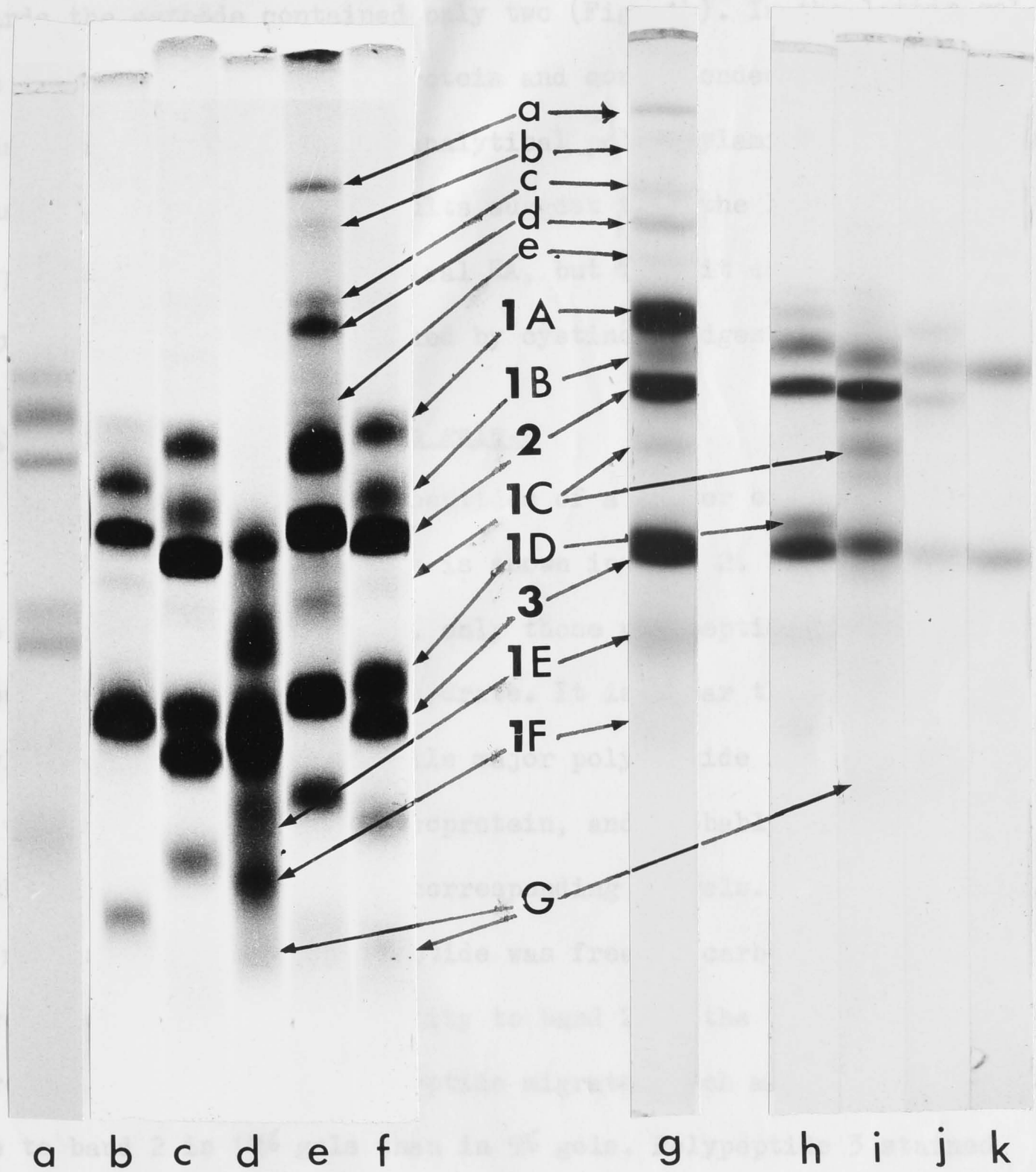


Fig. 1. Analysis in 5% polyacrylamide gels of various strains of influenza virus (a - i) or haemagglutinin fractions (j,k). The strains used were (a) A_2/SA/64, (b) A_2/NT/68, (c) $X7F_1$, (d) $RI/5^-$, (e,f) SW, (g - i) BEL, (j,k) BEL haemagglutinin fractions. Material was denatured with SDS at 100^o in 0.5 M-tris in the presence of dithiothreitol without (a - d,f, h - k) or with prior performic acid oxidation (d,i). G = glucagon.

tion to contain four polypeptides (Fig. 1i), while that migrating towards the cathode contained only two (Fig. 1k). In the latter gel, both bands stained for glycoprotein and corresponded in mobility to bands 1B and 1D found in the analytical polyacrylamide gels of whole virus (Fig. 1g, h). These results suggest that the largest major polypeptide was part of the viral HA, but that it contained two fragments of unequal size linked by cystine bridges.

Analysis in 10% polyacrylamide gels.

The pattern of polypeptides of a number of virus samples run in 10% polyacrylamide gels is shown in Fig. 2. When these gels were stained for glycoprotein, only those polypeptides indicated by arrows appeared to lack carbohydrate. It is clear that in 10% polyacrylamide gels the least mobile major polypeptide in denatured, but unreduced, virus is a glycoprotein, and probably corresponds to the 1A polypeptide in the corresponding 5% gels. After reduction, the most slowly moving major polypeptide was free of carbohydrate and corresponded in relative mobility to band 2 of the 5% gels. It seems, therefore, that the 1B glycopeptide migrates much more rapidly relative to band 2 in 10% gels than in 5% gels. Polypeptide 3 stained for glycoprotein only at its trailing edge, suggesting that polypeptide 1D was migrating with polypeptide 3. If virus, denatured with reduction, is prestained with Remazol Brilliant Blue R, this glycopeptide may occasionally be seen migrating just behind band 3, since the bound stain appears to reduce the mobility of the glycopeptide (Griffith, in preparation†). The polypeptide patterns obtained with

†Paper 2

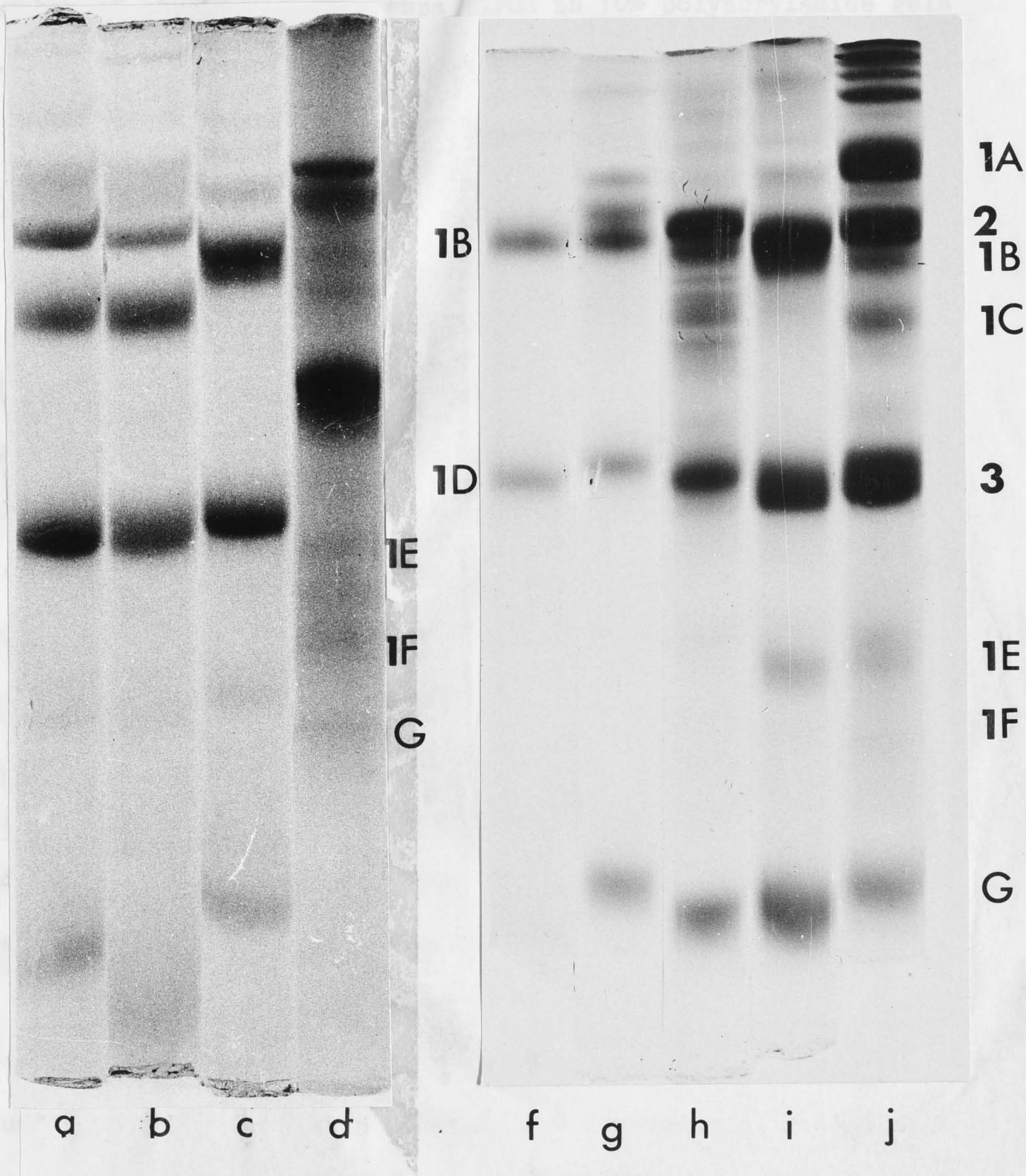


Fig. 2. Analysis in 10% polyacrylamide gels of various strains of influenza virus (a - e, h - j) or haemagglutinin fractions (f,g). The strains used were (a)A_2/NT/68, (b)X7F_1, (c - j) BEL, (f - g) BEL haemagglutinin fractions. Material was denatured with SDS at 100° in 0.5 M-tris in the presence of iodoacetamide (j), or in the presence of dithiothreitol without (a - i) or with prior performic acid oxidation (h). G = glucagon. Arrow = carbohydrate--free polypeptide.

a number of strains of influenza virus in 10% polyacrylamide gels are rather similar; the BEL strain, however, differs from the others in that its 1B polypeptide migrates more slowly in 10% than does that of other strains.

The molecular weights of the polypeptides.

The mobility of the SDS-protein complexes, both in 5% and 10% polyacrylamide gels, is proportional to the $\log_{10}$ of their molecular weights (Shapiro et al., 1967; Weber and Osborn, 1969; Dunker and Rueckert,1969). However, the mobilities of the two major glycopeptides of influenza virus denatured with reduction are obviously very different in the two types of gel. Thus, although the estimates of the molecular weights of the viral proteins (bands 2, 1c, 3) derived from 5%and 10% gels are in fairly good agreement (Table 1), the estimates for the viral glycoproteins are not. These findings suggest that this method of molecular weight estimation cannot be applied to glycoproteins.

Performic acid-oxidised virus appeared to lack the high molecular weight polypeptides, or where they were present, their amount (judged by intensity of staining) was reduced. At the same time, a number of minor polypeptides with a spectrum of molecular weights ranging from 12,000 to 40,000 were formed. It was possible that these represented oligomers of a simple low molecular weight polypeptide. This possibility was investigated using alternative and additional denaturing conditions. $X7F_1$ virus was used for this work, as its HA is readily denatured by SDS (Kilbourne et al., 1967).

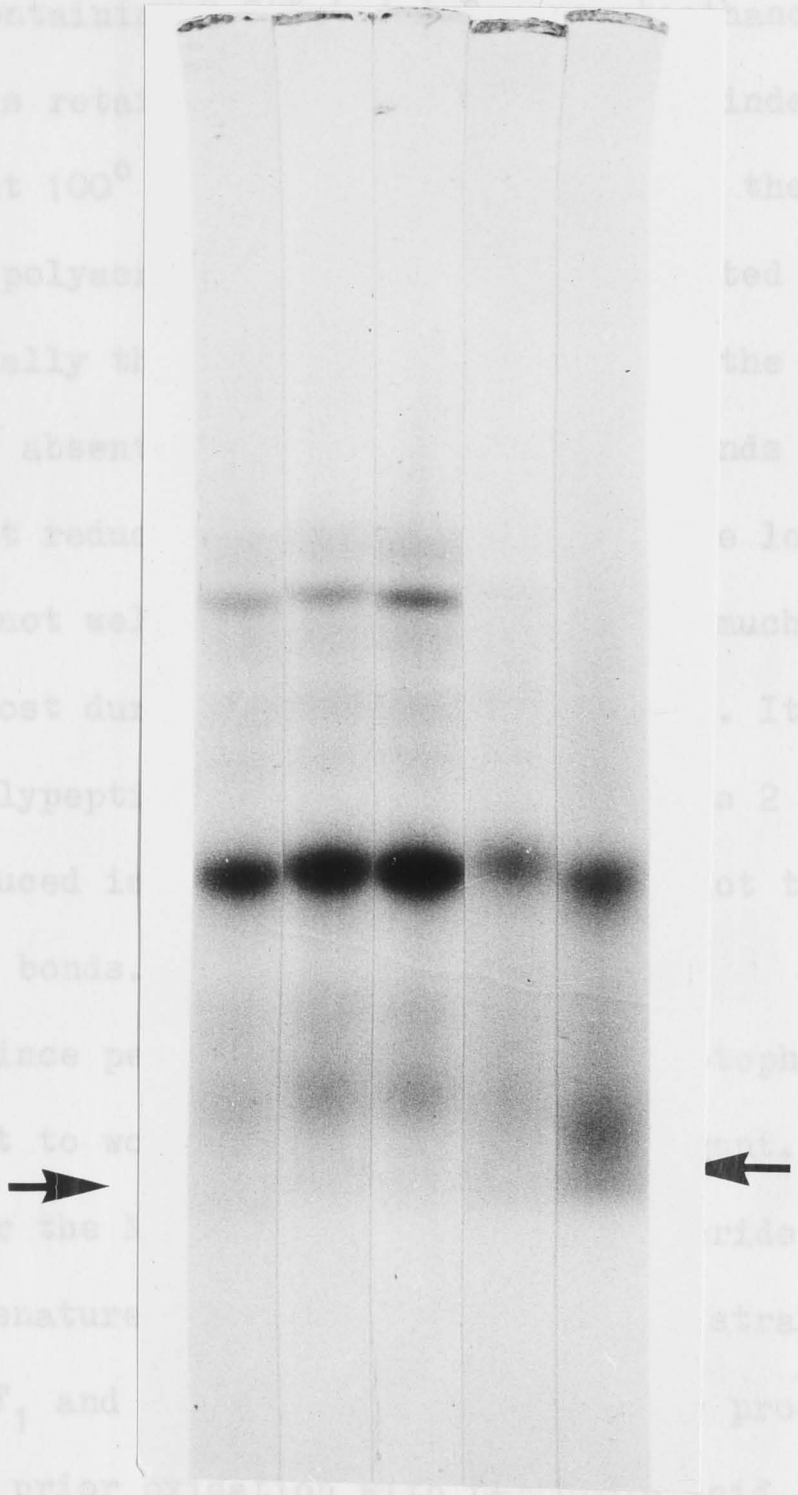
Result	Interpretation	Reinterpretation.	Valid			Comment
			Yes	Maybe	No	
Chapter 1	Antiserum specific for RNP	Specific for soluble antigens.	✓	✓	✓	RNP assumed to be the only internal antigen.
Chapter 2	RNP purified	Purified core antigen.		✓	✓	Probably another internal ('core) antigen; see Paper 1.
Chapter 3, upto p.79	Procedure for purification (a) some strains		✓	✓		Suitable for preparing pure virus, but not highly infectious virus. Superceded byPaper3
	(b) BEL				✓	
pp.79 - 86	Two density forms of influenza virus			✓	✓	May be valid for filamentous strains for spherical strains, see Paper 2,3. See Paper 1
		Aggregation with debris.	✓	✓		
Chapter 4 upto p.124	Ether and DOC do not degrade virus to uniform subunits		✓			
pp.124 - 134	SDS plus DOC gives two species of RNP			✓	✓	
		Gives two soluble antigens.	✓	✓		
	SDS does not reproducibly or completely disrupt BEL.		✓			See Papers, 1,3 and 5
	Fraction 3 can be a mixture of proteins.		✓			Compare Laver,(1964) and Laver and Webster (1966)
Fig. 4.12, 4.13	Fraction 1 is a mixture of proteins.			✓	✓	
		Two fragments of HA	✓			
Fig. 4. 16	Neuraminidase contains a highly mobile polypeptide in acrylamide gels.			✓		See Paper 5
						See Papers 1 and 5
Chapters 4, 5 Appendix	Gel system is best available. Virus is reduced to polypeptides by reduction with hot SDS at pH 9.0 and carboxymethylation in urea, and that the same polypeptides result if the virus is first oxidised with performic acid.				✓	Continuous buffer system reproducible Paper 4.
		Virus very resistant to denaturation.	✓		✓	See Paper 5
Fig. 5.4 Fig. 5.6	Peptide map of RNP R = RNP, H = HA N = neuraminidase	Peptide map or core antigen R = core antigen, H = RNP + HA	✓ ✓	✓ ✓ ✓	✓	Minor spots may be trace contaminants.
Fig. 5.7, Table 5.5	Molecular weights				✓	Paper 1,5; Taylor et al., (1969)
Table 5.6	Proportions of polypeptides				✓	Too high, incomplete denaturation — See Paper 5

Effect of high temperature and pH.

Performic acid-oxidised $X7F_1$ was subjected to 100° in the presence of SDS and DTT ~~at 100°~~ at five different pH values (Fig. 3). At pH 7.2, pH 8.0 and pH 9.0 no difference in polypeptide pattern was seen. Compared with performic acid-oxidised BEL, however, it had much less of bands 1B and 1C, and much more of bands 1E and 1F. At pH 10.0 and pH 10.7 band 1B was reduced, but band 2 was also missing. At the same time, more material migrating close behind the glucagon marker was present: it is likely that this represented low molecular weight polypeptides resulting from cleavage of peptide bonds of the high molecular weight polypeptides absent in these samples. It was concluded that denaturation at 100° at pH 10 and above was likely to be accompanied by cleavage of peptide bonds. These conditions were thus considered to be unsuitable.

Effect of NP 40 - guanidine hydrochloride - urea.

Performic acid-oxidised $X7F_1$ was reduced at room temperature for five hours with DTT in the presence of NP 40 and EDTA, in 0.25 M-$NaHCO_3$ saturated with guanidine hydrochloride and urea. The sample was then dialysed against 8 M-urea in 0.01 M-$NaHCO_3$, 0.1% NP 40 and 0.2% β-mercaptoethanol for 16 hours. Dialysis was continued against fresh solution containing 0.1% SDS in place of the NP 40. After 24 hours, the sample was reacted with iodoacetamide. A sample of this material was retained for gel analysis. The remainder was dialysed overnight against 0.01 M-sodium phosphate buffer



a b c d e

Fig. 3. Performic acid-oxidised X7F$_1$ virus was heated for 5 min at 100^o in the presence of SDS and dithiothreitol at (a) pH 7.2, (b) pH 8.0, (c) pH 9.0, (d) pH 10.0, (e) pH 10.7. Arrow = glucagon.

(pH 7.2) containing 0.01% (v/v) β-mercaptoethanol and 0.1% (w/v) SDS. A sample was retained for analysis; the remainder was heated for 5 minutes at 100° at pHs 7.0, 8.0 and 9.0 in the presence of DTT. Analytical polyacrylamide gels of virus treated in this way were all essentially the same (Fig. 4). Although the 1B band was much reduced, or absent, the other polypeptide bands were still present and were not reduced to smaller subunits. The low molecular weight bands were not well represented; presumably much of these polypeptides were lost during the prolonged dialysis. It was concluded that the polypeptides migrating between bands 2 and 3 could not be further reduced in size by any means known not to involve cleavage of covalent bonds.

Since performic acid destroys tryptophan, it is sometimes inconvenient to work with this denaturing agent. It was decided to test whether the NP 40 - guanidine hydrochloride - urea denaturing procedure denatured unoxidised virus. Three strains of virus (BVic65, $X7F_1$ and BEL) were denatured by the procedure both with and without prior oxidation with performic acid. The results are shown in Fig. 5. It is evident that none of the denatured, unoxidised samples had the same polypeptide pattern after denaturation as that virus which was first oxidised with performic acid. All the performic acid-oxidised samples had a greater content of the low molecular weight polypeptides, indicating that denaturation had been more complete with these samples. Even so, it was evident that with the BEL strain that some of the 1B band had resisted further

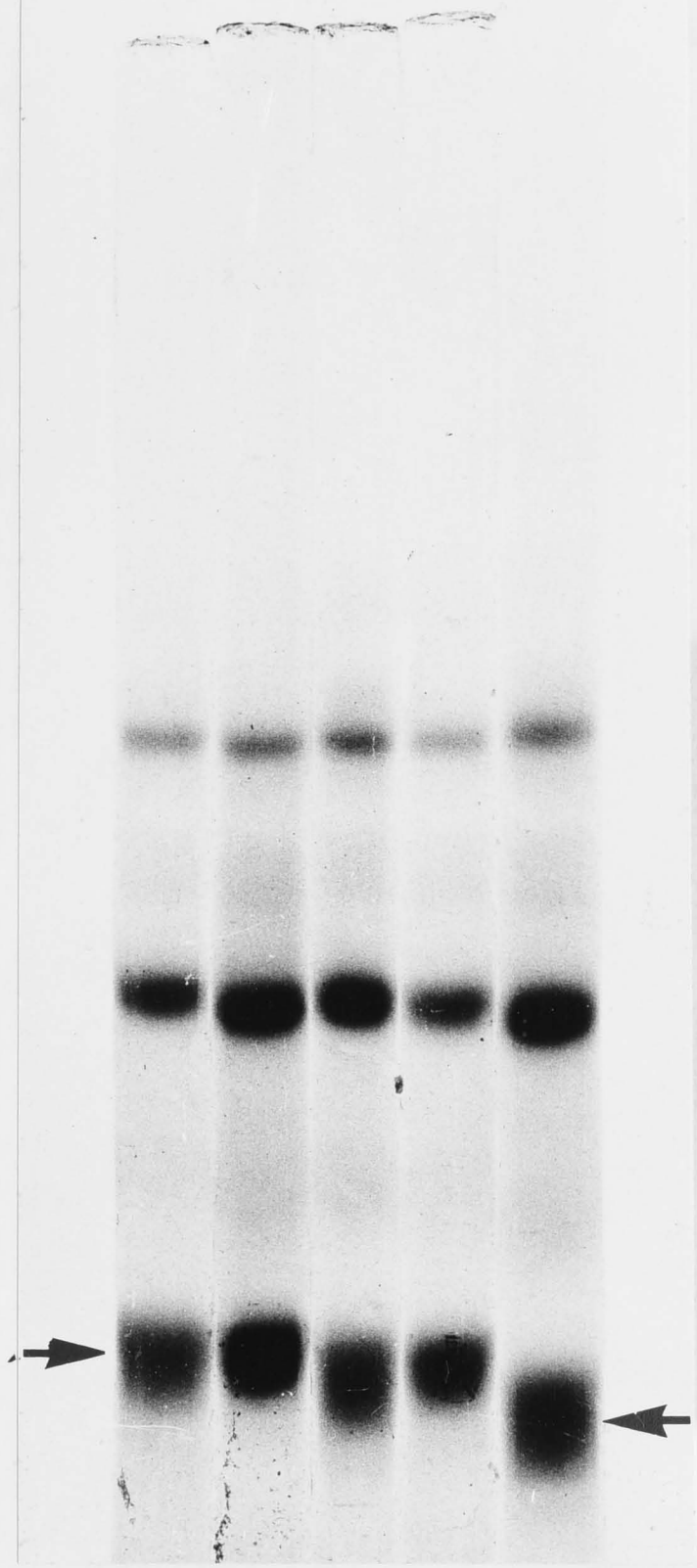


Fig. 4. Performic acid-oxidised $X7F_1$ virus was treated with NP 40, EDTA, urea and guanidine hydrochloride in 0.25 M-$NaHCO_3$ for 5 hours at room temperature, followed by dialysis first against urea, NP 40 and mercaptoethanol in 0.01 M-$NaHCO_3$, then against urea, mercaptoethanol and 0.1% SDS in 0.01 M-$NaHCO_3$. The sample was then carboxymethylated with iodoacetamide. (a) sample without further treatment, (b - e) sample dialysed against 0.1% SDS in 0.01 M-phosphate buffer, (b) not heated, (c - e) heated at 100° at pH 7.2 (c), 8.0 (d), 9.0 (e). Arrows = glucagon.

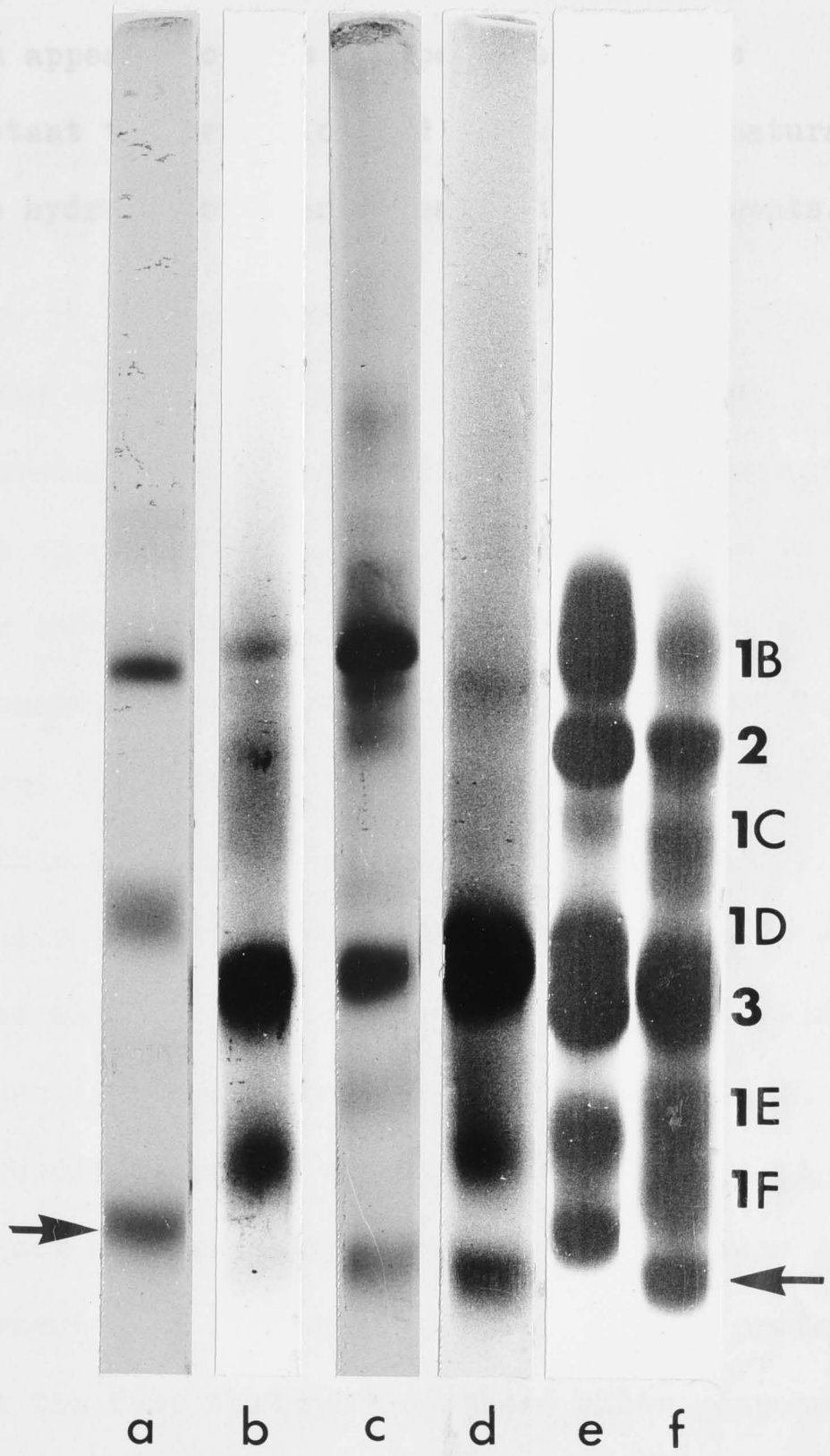


Fig. 5. Denaturation of three strains of influenza virus. (a,b) X7F$_1$, (c,d) B/Vic/65, (e,f) BEL. Virus was reduced at room temperature in NP 40-guanidine HCl-urea without (a,c,e) and with (b,d,f) prior performic acid oxidation. For details see text. Arrow = glucagon.

breakdown. This strain appears to have polypeptide complexes which are highly resistant to performic acid oxidation, to saturated solutions of guanidine hydrochloride and urea, and to detergents.

DISCUSSION.

The results presented in this paper show that influenza virus contains a number of major and minor polypeptide and glycopolypeptide components. To establish conditions under which virus was reproducibly reduced to its ultimate molecular components without cleavage of covalent bonds (other than disulphide bridges), it was considered more economical in both time and materials to use egg-grown virus rather than virus labelled in tissue culture with amino acids, and to locate the components by staining procedures. Furthermore, virus grown in tissue culture generally has a lower particle: ID_{50} ratio than that grown in embryonated eggs, and in any case, labelling fails to distinguish between virus-coded virion proteins and host-coded material induced by (or synthesised after) infection, and subsequently incorporated into the virion. It must be stressed that the results reported in this paper refer to virion components; these may not all necessarily be virus-coded. Some of the minor virion components may be passively incorporated, preformed host-cell protein, but the fact that most of these minor components were increased in amount by the various denaturation procedures, while the amount of major components were simultaneously decreased, suggests that the former were derived from the latter.

The possibility that some of these minor components resulted from nonspecific cleavage of polypeptides (except at 100° at pH 10 or higher) was not excluded but is considered unlikely. Treatment of a number of standard proteins with tris base at 100° does not

lead to obvious cleavage of the polypeptide (Griffith, in preparation[+]). However, glycoproteins are known to be degraded at elevated temperatures and alkaline pH. Cleavage of the polypeptide chain by β-elimination (Neuberger and Marshall, 1966) is probably prevented by the presence of sulphydry compounds during denaturation, since these would react with the α-β unsaturated derivatives resulting from β-elimination of serine- or threonine-linked carbohydrate residues. However, the loss of large pieces of carbohydrate from the virion glycoprotein, by B-elimination, or scission of alkali labile 1 → 3 glycosidic linkages is not excluded in the present work, especially with the high temperature-tris denaturation procedure; however, it is unlikely to have occurred during denaturation with 0.25 M-$NaHCO_3$ at room temperature. The minor polypeptide components formed during the latter denaturation procedure are thus considered to be derived from the higher molecular weight major components by cleavage of disulphide bridges.

Proteins migrate at a rate proportional to their molecular weights in SDS-polyacrylamide gels (Shapiro et al., 1967); a variety of acrylamide concentrations may be used. The molecular weight of a protein may be calculated from its mobility in SDS-polyacrylamide gels by comparison with standard proteins of known molecular weight electrophoresed in gels of the same acrylamide concentration (Dunker and Rueckert, 1969). It is clear from the results presented in Table 1 that the influenza A strains contain two polypeptides (bands 2 and 3)which give molecular weights of about 45,000 (band 2) and 22,500(band 3) in both 5% & 10% polyacrylamide gels. The periodic acid-Schiff stain did not

[+]Paper 4

reveal the presence of any carbohydrate in these polypeptides. In contrast, the other polypeptides of influenza virus did contain carbohydrate. None of these migrated at the same rate (relative to standard proteins) in both 5% and 10% gels. The listed molecular weights are therefore the apparent rather than the real molecular weights. The intensity of the Schiff stain was greatest in those bands (other than band 2) migrating with apparent molecular weights greater than 30,000 in 5% gels. The difference between the mobilities of the 1B bands in 5% and 10% gels is especially obvious, giving apparent molecular weights of about 55,000 in 5% gels and 44,000 (BEL) or 37,000 (others) in 10% gels. The differences in their mobilities in the 5% and 10% gels of the other major polypeptides is less marked, but still significant. The reason for this anomalous behaviour is almost certainly due to the presence of carbohydrate in these polypeptides.

It is probable that the viral haemagglutinin is composed of an aggregate of three of the 1A polypeptides (Griffith, 1969; Griffith and Wrigley, in preparation[+]). Each of these seems to consist of two portions relatively easily separated into the 1B and 1D polypeptides by denaturation with SDS at 100° and pH 9.0 under reducing conditions. Failure to separate these two portions by denaturation without reduction suggests that they are linked by cystine bridges. It is unlikely that a change in mobility resulting from unfolding of polypeptide 1A following reduction of cystine bridges was responsible for band 1B, since reduction of cystine

[+]Paper 1

bridges in proteins with molecular weights of this order is accompanied by a reduction in mobility in SDS-polyacrylamide gels (Griffith, in preparation[+]). The 1D polypeptide is quite resistant to further denaturation by reduction, but its absence in samples of virus oxidised with performic acid suggests that it too is composed of subunits linked by cystine residues resistant to reduction, but susceptible to oxidation. Likewise, the 1B polypeptide appears to be at least partially susceptible to further degradation, relatively easily accomplished with the haemagglutinin of $X7F_1$ and $RI/5^-$, but less readily with that of BEL; indeed, that of BEL appears to be at least partially resistant to oxidation followed by reduction in the saturated guanidine hydrochloride - urea solution.

The markedly anomalous behaviour in the two gel systems of the 1B glycopolypeptide suggests that it contains a higher percentage by weight of carbohydrate than the other major glycopolypeptides. The minor polypeptides between band 2 and 1D (5% gels) stain well for carbohydrate and poorly for protein. It is likely that these are derived at least in part from the 1B glycopeptide. The fate of the 1D polypeptide on oxidation is not clear, but it may consist of smaller polypeptides linked by oxidation-susceptible cystine residues. The marked difference in mobility in 10% gels between the 1B bands of BEL and other strains was the only significantly different behaviour between analagous polypeptides of the different strains. The reason for this is not clear, but the high resistance of the HA of this strain to SDS denaturation (Laver, 1964) suggests that

[+] Paper 4

structurally it is somewhat different from that of other strains. Insufficient data were available to determine whether the small differences in the molecular weights of equivalent polypeptides of the different strains were significant. It does appear, however, that the B/Vic/65 strain does have somewhat larger polypeptides 2 and 1D than the A strains.

The extreme resistance of a portion of the 1A band in all strains suggests that another virion polypeptide may be migrating together with the unfragmented HA polypeptide. This polypeptide has an apparent molecular weight of about 55,000 (10% gels) or 62,000 (5% gels). Isolated neuraminidase has been shown to have a molecular weight of this order (Rafelson et al., 1966; Webster, 1970; E.A. Haslam, personal communication). From its dimensions in electron micrographs, the 'head' of the neuraminidase molecule has been calculated to have a molecular weight of 64,000, possibly composed of four subunits of molecular weight 16,000 (Griffith and Wrigley, in preparation+). It seems possible that this portion of the 1A polypeptide resistant to denaturation is in fact derived from the neuraminidase. Whether it (like the HA) also eventually breaks down into subunits has yet to be firmly established.

The smaller of the two polypeptides lacking carbohydrate (band 3) has not yet been identified with any of the known viral antigens; it has been suggested that it is a core protein involved in 'packaging' the RNP coil within the virion (Griffith and Wrigley, in preparation+). The identity of the larger polypeptide (band 2) lacking carbohydrate has been established; it is derived from the

+ Paper 1

influenza virion RNP (Taylor et al., 1969; White et al., 1970; Pons et al., 1969; Joss et al., 1969); the more rapid migration of the 1B polypeptide in 10% gels established why different workers identify the RNP with the major polypeptide of second lowest mobility in 5% gels (White et al., 1970), and with the major polypeptide of lowest mobility in 8% (Pons et al., 1969) or 10% gels (Joss et al., 1969). The results reported here also indicate that the assumption of identity of the various peaks obtained in polyacrylamide gels of different concentrations (Dimmock and Watson, 1969) may be in error. The results reported here also suggest that several very high molecular weight polypeptides (Dimmock and Watson, 1969) in influenza virus preparations may be a result of incomplete denaturation. Dimmock and Watson (1969) also used a discontinuous buffer system, and the smaller polypeptides present in their preparations may have 'stacked' at the buffer front. In any case, it is possible that their molecular weight values are inaccurate, as it has not been shown that a linear relationship exists between the mobility in polyacrylamide gels of SDS-charged polypeptides and the $\log_{10}$ of their molecular weights using discontinuous buffer systems.

Minor components reported by other workers to be present in the virus are probably a result of partial breakdown of the HA, and possibly the neuraminidase. The results reported here indicate that the minor components vary in amount and mobility depending upon the denaturation procedure used. Furthermore, the amounts present in different virus preparations vary (Pons and Hirst, 1969). The

presence of these minor components in highly purified BEL and SW virus preparations which have been denatured without reduction, suggests that they may normally be present in virions. The results reported here and elsewhere (Laver and Valentine, 1969; Griffith and Wrigley, in preparation) indicate that the viral neuraminidase and HA are highly complex structures. How the infected cell correctly assembles these structures is not known, but 'mistakes' may be made and improperly assembled subunits less resistant to denaturation may be normally present in virions. 'Mistakes' in disulphide bridging of the subunits might explain the presence of small amounts of the polypeptides (all glycopeptides) with molecular weights greater than 70,000. Gross errors may lead to the formation of 'incomplete' virus; it is of interest in this respect that non-haemagglutinating, non-infectious, low density, membranous material present in the allantoic fluid of BEL-infected eggs appears to contain all the virion polypeptides (Griffith, unpublished observations). The use by many workers of virus preparations of low infectivity grown in tissue cultures (to permit labelling of the viral polypeptides) may be one explanation for the presence of these minor components in their virus preparations.

+Paper 1

++ Paper 3

PAPER 6.

A simple procedure for the recovery of protein from preparative gel slabs.

The vertical slab apparatus described in chapter 5 has been modified for use with 5% acrylamide gels and 0.1 M-phosphate buffer (pH 7.2) containing 0.1% SDS and Tris-thioglycollate (pH 7.0, 0.01 M). As this buffer has a poor buffering capacity, it has to be recirculated with a pump. The electrodes are inserted into dialysis tubing; buffer in the electrode compartments so formed, is constantly recirculated from one electrode to the other with a peristalic pump.

Protein bands in the acrylamide gel slabs may be simply and rapidly located using the fluorescent stain, magnesium 1-anilino-8-naphthalene sulfonate (Hartman and Udenfriend, 1969).

The protein recovery method is essentially that described in chapter 5, but with some modifications. Gel fragments are mounted in 4% polyacrylamide gel, buffered with 0.01 M phosphate buffer pH 7.0. The lower reservoir buffer (tris-glycine) is replaced by 0.1 M sodium phosphate (pH 6.0) containing 0.1% SDS and tris-thioglycollate (0.01 M, pH 7.0). The upper reservoir contains 5% ammonium carbonate, and tris-thioglycollate (pH 7.0, 0.01 M). The whole recovery assembly is placed in a polythene bag and immersed in ice-water during electrophoresis of protein out of gel fragments.

Recovered protein is dialysed for a few hours against a large volume of water to remove most of the salts. The volume of fluid in the dialysis bag is reduced by pervaporation to about 5 ml.

and the protein precipitated from this by adding 100 ml acetic acid : methanol : acetone (1 : 12 : 24 by volume).

Precipitated protein is dissolved in the minimum quantity of formic acid : pyridine (19 : 1 by volume) and applied to a column of Sephadex LH 20 (25 x 1.5 cm) equilibrated with the same solvent. Fractions containing the protein are identified by placing a drop of each on filter paper and staining for protein with Procion blue (this of course is unnecessary with Remazol stained protein). Fractions containing protein are pooled and the protein recovered by precipitation with water : methanol : acetone (1 : 6 : 12 by volume). This desalting procedure is more rapid and effective than that described in chapter 5. Less than 0.001% of added radioactive amino acids are carried through this procedure.

The recovery of Remazol stained standard proteins is virtually quantitative. The procedure and recoveries have yet to be checked with unstained protein. For this viral protein labelled with radioactive iodine will be used.

GENERAL DISCUSSION OF RESULTS.

DISCUSSION.

The question as to whether there are one or two (or maybe even more than two) internal antigens in the influenza virion has yet to be clearly established. However, there are a number of reasons to suppose that at least two exist, although the evidence for the existence of a second internal antigen is at present rather circumstantial. If we are to accept that a 'core' antigen exists, the following observations may be explained:

1) The presence of a second internal component distinct from the RNP as is seen in electron micrographs of influenza virus (Paper 1; Bächi et al., 1969; Compans and Dimmock, 1969; Apostolov, et al., 1970).

2) The migration of two polypeptides from the nucleus to the cytoplasm of infected cells and subsequently into the virion (Taylor et al., 1969; Joss et al., 1969; White et al., 1970).

3) The presence of a "soluble antigen" with peptide maps quite different from those of the protein assumed to be the RNP.

4) The difficulty encountered in purifying the "soluble antigen" using an assay of assumed specificity.

Before a protein may be isolated from a complex mixture in a pure form, it is essential that an assay procedure is available which is specific for the protein being isolated. To assume an assay is specific is inadvisable. Unfortunately, it is impossible to prove an immunological assay is specific unless pure protein is available to test it. The vicious circle which such a situation engenders is

admirably demonstrated in Chapters 1 and 2 of this work. The use in this work of the term 'RNP' as being synonomous with 'soluble antigen' is probably incorrect. Indeed, if the whole of Chapters 1 and 2 are read with the term 'a soluble antigen' in place of 'RNP', the results may be more meaningful. If two soluble antigens exist, it is unlikely that preparation of crude extracts by precipitation at pH 4.6 included one but not the other antigen, although one may be preferentially concentrated by this procedure; indeed, the immunodiffusion tests in Chapter 1 indicated that one soluble antigen (or antibody to it) was present in greater amounts than the other in the reagents used. It is likely that antibodies to both the RNP and postulated core antigen exist in the immune sera prepared as described in Chapter 1. If this is so, it is not surprising that difficulty was encountered in recovering the soluble antigen in high yields from infected tissues (Chapter 2) or virus (Chapter 4). Moreover, the isolation from disrupted virus of two bands of protein (bands 4 and 3, Fig. 4.7) reacting in the complement fixation tests may now be explained; one is presumably the RNP and the other the core antigen. The specificity, or lack thereof, of the sera prepared still awaits purification of the soluble antigens. It was considered unwise to continue work with, or on, these sera, or on the preparation of "soluble antigen" from infected tissues, until it was shown whether the assumption that a single internal antigen existed was correct. The obvious way to tackle the problem would be to disrupt purified virus and separate

the components by polyacrylamide gel electrophoresis and to establish their number and identity.

The work described in Chapters 1 and 2 should thus be reconsidered with these problems and possibilities in mind. Any future procedure for the preparation of pure 'core' antigen, and for the preparation of specific immune serum to this antigen, may well follow the pattern described in Chapters 1 and 2; this work may be considered now as sighting experiments for this project. A more satisfactory initial assay procedure for the purification of the core antigen is undoubtedly the use of analytical polyacrylamide gels, since we may now estimate the content of core antigen in fractions by comparison with analytical gels of whole virus. This procedure would naturally be simplified by the rapid pre-staining technique described in Paper 2 of this addendum. Once purified, the core antigen could be used to prepare specific antisera by immunisation of rabbits; the production and testing of such sera would be essentially as described in Chapter 1.

The low RNA content of the soluble antigen purified in Chapter 2 suggests that it was the proposed core antigen which was in fact purified. Although this protein was probably impure (hence the presence of RNA), the large number of neutral and basic peptides present indicates some similarity between this protein and the "R" polypeptide isolated and mapped in Chapter 5 (Fig. 5.4). A comparison of the peptide maps of BEL fraction 3 prepared at different times by Laver (Laver, 1964; Laver and Webster, 1966) reveals that

many of the additional peptides in the later map (Laver and Webster, 1966) are neutral peptides. It is probable that this worker mapped one antigen on the first occasion and two (or more) on the second. Of the two polypeptides which accumulate in the nucleus of infected cells (Taylor et al., 1969), the one with the higher molecular weight corresponds to the RNP (White et al., 1970); the one with the lower molecular weight, corresponds to the "R" polypeptide; the latter had many neutral and basic peptides (Fig. 5.4). It seems likely that the earlier peptide map of Laver was of the RNP, and the later one was in fact of a mixture of the RNP and the antigen containing the neutral peptides, namely the core antigen. The small differences observed in the peptide maps of Fraction 3 (or 1) of different virus strains which are claimed to be differences in the RNP's of the strains (Laver, 1964; Laver and Kilbourne, 1966; Webster et al., 1968) may therefore in fact be due to varying amounts of different viral antigens in the "RNP" preparations. This point was made in Chapter 4 when discussing the variable resistance of influenza virus to disruption with detergents (Fig. 4.8, 4.9, 4.12), the presence of more than one polypeptide in Fraction 3 proteins (Fig. 4.11, 4.12), and when comparing peptide maps of the Fraction 3 proteins of four strains (Fig. 4.10). This work is considered of value as it explains the inconsistencies in the maps of Laver, and emphasises the difficulty of interpreting peptide maps performed on mixtures of proteins.

The virus purification procedure previously described in

Chapter 3 (pages 70 - 79) is considered to be valid, as it has been used to prepare the virus described in Paper 1, and (except for the use of two cycles of adsorption-elution with human erythrocytes) for the preparation of the virus strains (other than BEL) examined in Paper 5. The whole of Chapter 3 illustrates that red-cell adsorption-elution may introduce more problems than it solves. This applies especially to certain strains (e.g. BEL). The glycerol-sucrose-tartrate gradient described in Paper 3 separates virus from red-cell debris in much the same way as the sucrose-tartrate gradients described in Chapter 3. Even after prolonged centrifugation, the band of red-cell debris in these gradients also contains much haemagglutinin. However, virus prepared by adsorption-elution of allantoic fluid with aluminium phosphate has very little of this low density haemagglutinin (paper 3). Since treatment of the red-cell debris band with Tween 80 releases only a portion of the virus in this band, it is likely that the low density haemagglutinin was in fact virus firmly adsorbed to or even fused with, red-cell debris. It is now considered that the host cell antigens and density heterogenity of the virus examined in Chapter 3 (pages 79 - 86, Figs. 3.7 - 3.17) resulted from aggregation of virus with red-cell debris, and thus this work is considered to be of no importance.

It is evident from the work of others that the most slowly migrating <u>major</u> polypeptide in 10% gels and the second most slowly migrating <u>major</u> polypeptide in 5% gels is that derived from the RNP

(Joss et al., 1969; White et al., 1970). Thus, the assumption in Chapters 4 and 5 that the polypeptide R was derived from the RNP is wrong. As discussed above, it is probably derived from the proposed core antigen. The major polypeptide of lowest mobility in Fig. 4.12 and 4.13, which was proposed as part of the haemagglutinin, has since been stained with the periodic acid-Schiff reagent and is considered to be definitely part of the viral haemagglutinin. Likewise, the other major polypeptide present in the haemagglutinin fractions (Fig. 4.12, samples 1a, 1b, 0) is also a glycoprotein. This component of the haemagglutinin runs very close to the core polypeptide both in 5% and 10% gels, and it is likely that this polypeptide was running together with the "R polypeptide" (Fig. 4.13), and was not resolved by the 8% acrylamide gels containing urea used in earlier work. The virus used for this work had not been reacted with performic acid, but denatured with SDS under reducing conditions at pH 8.9 and 100°, thus corresponding to virus denatured under similar conditions in Paper 5. It may now be stated that the two major polypeptides present in the haemagglutinating fractions (Fig. 4.12) are both glycoproteins derived from the viral haemagglutinin. The earlier assumption that the smaller of these polypeptides was part of the RNP is wrong. It was not seen in analytical polyacrylamide gels of virus oxidised with performic acid, and this compounded the misunderstanding.

The identity of the polypeptide N is still unresolved. Webster (1970) and Haslam (personal communication) have indicated

that the polypeptide derived from the neuraminidase has a molecular weight close to 60,000. Neither of these workers used performic acid to oxidised virus; performic acid was used on the virus and protein fractions analysed in the gels shown in Figs. 4.16 and 5.6. As the polypeptides which migrate more slowly than the RNP polypeptide are reduced to subunits by the performic-oxidation plus saturated guanidine hydrochloride etc. denaturation procedure, it is possible that these workers were in fact looking at intact neuraminidase heads. The molecular weight of the neuraminidase subunit estimated from the dimensions of the protein (Paper 1) corresponds closely to that for the polypeptide N (Fig. 5.7). However, the certain identity of this polypeptide awaits analysis of $X7F_1$ fractions after <u>total</u> denaturation and analysis in 5% Maizel gels. If we accept that the head of the neuraminidase consists of four subunits linked by cystine bridges which are extremely resistant to reduction, but susceptible to performic acid oxidation followed by reduction, then the identification of the neuraminidase with this highly mobile polypeptide is probable.

In contrast to virus denatured with reduction, performic acid-oxidised virus does not contain the haemagglutinin component migrating just behind the proposed "core" polypeptide. Since the polypeptides isolated from the preparative gels (Chapter 5) were oxidised with performic acid, it is likely that the core polypeptide (previously referred to as the "R" polypeptide) was not contaminated with substantial amounts of haemagglutinin protein. Thus, the peptide

map of this polypeptide is still valid. That of the H polypeptide was probably a mixture of the RNP polypeptide and the portion of the haemagglutinin resistant to oxidation, and may be considered to be no longer valid. For the same reason, the amino acid analyses of the H polypeptides are also invalid. Whether the highly mobile polypeptide (N) was derived from the neuraminidase alone, or was a mixture of a number of polypeptides "stacked" at the buffer discontinuity, has not been ascertained. The small amount of stained material evident in these bands (Fig. 5.3) suggests that the latter possibility is unlikely. However, viral lipid migrated just ahead of this band, and the presence of ninhydrin-staining material (e.g. serine, ethanolamine, choline, sphingosine) in this lipid might complicate amino acid analyses. For the same reason, the analyses performed on whole virus (which was not defatted) are unlikely to be accurate; the analyses were in fact performed to allow an estimation of glucosamine content of whole virus. All amino acid analyses were performed on a Beckman analyser using the expanded scale which gives an accuracy of, at best, 10%; this was because most of the hydrolysed samples were lost in an accident involving a technician. The amino acid analyses must therefore be regarded as approximations of the amino acid content of the polypeptides R and N; that of the H polypeptide is invalid as mentioned above. The molecular weight estimations in Chapter 5 can now be replaced with those given in Paper 5. The techniques of isolation of the viral polypeptides are essentially as in Chapter 5, with the modifications outlined in

Result	Interpretation	Reinterpretation.	Valid			Comment
			Yes	Maybe	No	
Chapter 1	Antiserum specific for RNP	Specific for soluble antigens.	✓	✓	✓	RNP assumed to be the only internal antigen.
Chapter 2	RNP purified	Purified core antigen.		✓	✓	Probably another internal ('core) antigen; see Paper 1.
Chapter 3, upto p.79	Procedure for purification (a) some strains		✓	✓		Suitable for preparing pure virus, but not highly infectious virus. Superceded byPaper3
	(b) BEL				✓	
pp.79 - 86	Two density forms of influenza virus			✓	✓	May be valid for filamentous strains for spherical strains, see Paper 2,3.
		Aggregation with debris.	✓	✓		See Paper 1
Chapter 4 upto p.124	Ether and DOC do not degrade virus to uniform subunits		✓			
pp.124 - 134	SDS plus DOC gives two species of RNP			✓	✓	
		Gives two soluble antigens.	✓	✓		
	SDS does not reproducibly or completely disrupt BEL.		✓			See Papers, 1,3 and 5
	Fraction 3 can be a mixture of proteins.		✓			Compare Laver,(1964) and Laver and Webster (1966)
Fig. 4.12, 4.13	Fraction 1 is a mixture of proteins.			✓	✓	
		Two fragments of HA	✓			
Fig. 4. 16	Neuraminidase contains a highly mobile polypeptide in acrylamide gels.			✓		See Paper 5
Chapters 4, 5 Appendix	Gel system is best available. Virus is reduced to polypeptides by reduction with hot SDS at pH 9.0 and carboxymethylation in urea, and that the same polypeptides result if the virus is first oxidised with performic acid.					See Papers 1 and 5
					✓	Continuous buffer system reproducible Paper 4.
					✓	See Paper 5
		Virus very resistant to denaturation.	✓			
Fig. 5.4	Peptide map of RNP	Peptide map or core antigen			✓	
Fig. 5.6	R = RNP, H = HA	R = core antigen,	✓	✓		Minor spots may be trace contaminants.
	N = neuraminidase	H = RNP + HA		✓		
				✓		
Fig. 5.7, Table 5.5	Molecular weights				✓	Paper 1,5; Taylor et al., (1969)
Table 5.6	Proportions of polypeptides					Too high, incomplete denaturation — See Paper 5

Paper 6.

The results, and the interpretation of the results, of the original submission which may be regarded as valid, probably valid, possibly valid, or invalid, are indicated in the accompanying Table.

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